REMARKS/ARGUMENTS

I. Status of the Claims

After entry of this amendment, claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-55, 65-68, 70-77, 79-86, and 87-106 are pending. Claims 1-2, 31, 34, 52, 54-55, 66-68, 70-77, 79-86 have been amended. Claims 5, 7, 9, 18, 37, 39, 41, 50, 56-65, 69, and 78 are cancelled. Claims 22-30 are withdrawn. Claims 87-106 are newly added.

II. The Invention

It is the discovery of the present inventors that fucosyltransferases can be used *in vitro* to modify glycosylation patterns of glycopeptides. Accordingly the examined claims are drawn to *in vitro* methods for modifying glycosylation patterns of glycopeptides, including recombinantly produced glycopeptides using fucosyltransferases, especially fucosyltransferases that lack their membrane anchoring domain.

III. Support for the Amendments

Claims 1-2, 31, 34, 52, 54-55, 66-68, 70-77, 79-86 have been amended to recite that the claimed methods are carried out "*in vitro*". Support for this amendment can be found, *inter alia,* at page 27, lines 9-15, the example section in the specification and the title of the application.

New claims 87-106 have been added to set forth certain detailed aspects of the methods for large scale production of glycopeptides having a desired fucosylation pattern. Support can be found, *inter alia,* in claims as originally filed and on page 19, lines 32-35 and page 20, lines 1-5 in the specification.

These amendments do not introduce any new matter. Applicants respectfully request entry of these amendments.

IV. The Responses to the Rejections

Under 35 U.S.C. § 112, First Paragraph, enablement

Claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-55, 65-68, 70-77, and 79-86 are rejected under 35 U.S.C. § 112, first paragraph as allegedly not enabled.

Applicants respectfully submit that the scope of the claims at issue is fully supported by the disclosure provided in the specification. The present invention is directed to using fucosyltransferases *in vitro* to obtain a substantially uniform fucosylation pattern for glycopeptides. According to the specification teaching, fucosyltransferases useful for the *in vitro* glycosylation methods provided by the present invention can be any fucosyltransferases known or publicly available to one skilled in the art. For example, the specification teaches that "fucosyltransferases include any of those enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar." (page 26, lines 2-3 in the specification).

The specification further describes numerous examples of fucosyltransferases suitable for the present invention including Galβ(1→3,4)GlcNAc α(1→3,4)fucosyltransferase (FucIII E.C. No. 2.4.1.65), βGal(1→4)βGlcNac α(1→3)fucosyltransferases (FucT-IV, FucT-V, FucT-VI, FucT-VII, E.C. No. 2.4.1.65), α1,2 fucosyltransferase (E.C. No. 2.4.1.69), and α1,3 fucosyltransferase IX. See page 26 lines 1-29 in the specification. In addition, the specification provides a table of exemplary fucosyltransferases useful for *in vitro* methods of the present invention including FucT-III, IV, V, VI, and VII along with their respective tissue distribution patterns, substrates, and products.

The specification also teaches certain specific fucosyltransferases, *e.g.,* FucT-VII and FucT-VI that are capable of catalyzing at least 60% of their targeted glycopeptide-linked fucosyltransferase acceptor sites present in a population of glycopeptides. See page 27, lines 5-14 in the specification.

In addition, the specification teaches that fucosyltransferases of the present invention can lack the membrane anchoring domain. See page 12, lines 10-15 in the specification. As acknowledged by the Office Action, fucosyltransferases including their sequence information are known and publicly available. One skilled in the art can readily identify the stem region, trans-membrane region and the C-terminal region of a given fucosyltransferase based on its amino acid

sequence information, *e.g.,* via hydropathy plots. See also page 443-444 Molecular Biology of the Cell, second edition published by Garland Publishing, Inc. A copy of which is attached as Exhibit A. Therefore, one skilled in the art can easily identify and delete the membrane anchoring domain, *i.e.,* the trans-membrane region of a fucosyltransferase for it to be used in the present invention.

The example section of the specification further demonstrates that by following the teaching of the present invention, one can use FucT-V and FucT-VI to achieve a substantially uniform fucosylation pattern for a desired glycopeptide *in vitro*. Specifically Example 1 demonstrates that the fucosylation reaction using FucT-VI was completed in less than 24 hours and achieved a substantially uniform fucosylation pattern. Example 2 demonstrates that FucT-VI was capable of incorporating approximately 8-fold more fucose than FucT-V. Example 3 demonstrates that a fucosylation step can be proceeded by a sialylation step as taught by the present invention.

In addition, Example 4 demonstrates that additional fucosyltransferases useful for the present invention can be readily identified by using a simple radiolabeling method widely used in the field. Specifically a tracer amount of radiolabeled GDP-fucose can be added to a fucosylation reaction, which can be subsequently used to determine the amount of fucose incorporated into a protein, *e.g.,* by quantifying the radioactivity using an in-line scintillation detector.

Furthermore, by following the *in vitro* fucosylation method taught by the present invention, a research group including the inventors of the present invention has successfully demonstrated the use of soluble human FucT-VI, *i.e.,* FucT-VI without the transmembrane domain to produce a therapeutic peptide (complement inhibitor) with a substantially uniform glycosylation pattern and such achievement has been accepted and published in the journal of Glycobiology. A copy of which is attached as Exhibit B.

In summary, the specification clearly teaches that any fucosyltransferases can be used *in vitro* to provide a substantially uniform glycosylation pattern for glycopeptides. It enumerates a sufficient number of readily available fucosyltransferases including preferred fucosyltransferases

that can be useful for the methods provided by the present invention. The specification also provides ample examples to demonstrate the operability and success of the claimed methods achieved by following the teaching and guidance provided by the present invention. In addition, the published study directed to obtaining uniform fucosylation pattern via practicing the methods taught by the present invention is a further validation of the enabling teaching provided by the present invention.

The Office Action seems to suggest that not all fucosyltransferases can be used *in vitro* to obtain a substantially uniform glycosylation pattern for glycopeptides, therefore one skilled in the art has to determine which fucosyltransferases has the ability to provide substantial uniform glycosylation *in vitro*. Applicants respectfully submit that the specification clearly teaches that any fucosyltransferases can be used in the *in vitro* methods provided by the present invention. It also identifies certain specific fucosyltransferases, *e.g.*, FucT-IV, V, VI, and VII as preferred fucosyltransferases to be used in the *in vitro* methods and further describes that deletion of the membrane anchoring domain is also preferred for the *in vitro* methods provided by the present invention.

To the extent that the enablement rejection is in fact based on skepticism about the invention, Applicants point out that statements of generic operability in the specification must be accepted as accurate in the absence of proof to the contrary. Wettstein v. Campbell 139, USPQ 341, 343 (BOPI 1962). Thus, whenever a rejection is based on the contention that the disclosure is not commensurate in scope with the subject matter being claimed, "it is incumbent upon the Patent and Trademark Office.... to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement (emphasis added)." In re Marzocchi 169 USPQ 367, 369-370 (CCPA 1971).

In the present case, the Office Action has clearly failed to provide any evidence or sound reasoning in support of its contention that not all fucosyltransferases including the preferred fucosyltransferases can be used in the *in vitro* methods provided by the present invention.

Therefore, in the absence of any supporting evidence to the contrary the specification teaching must be taken as credible and in compliance with the enabling requirement.

Furthermore, Applicants respectfully point out that the claimed invention is directed to an *in vitro* method using widely known and well studied fucosyltransferases. According to the present invention, the fucosyltransferases useful for the *in vitro* methods of the present invention include any wild type fucosyltransferases or equivalents thereof, *e.g.,* fucosyltransferases containing modifications insubstantial to their fucosylation activity. One skilled in the art has extensive knowledge about the structure of fucosyltransferases including its highly conserved amino acids, binding sites for GDP-fucose, catalytic domain, etc. See also a copy of an article published in the Journal of Biological Chemistry attached as Exhibit C. This article, especially the introduction section is an example of the extensive knowledge readily available to the public about the structure and function of members of the fucosyltransferase family. Based on the information available in the field, it is well within the ability of one skilled in the art to understand which region(s) are important for the fucosylation activity, and thus should not be modified in order to preserve the desired fucosylation activity. In addition, should one choose to use a fucosyltransferase modified within highly conserved region(s), he or she can easily determine whether such modified fucosyltransferase is useful for the *in vitro* methods of the present invention, *e.g.,* by using the radiolabeling method taught and demonstrated by the present invention.

Therefore, the enablement rejection is without merit and should be withdrawn. It has long been held by the Court of Appeals for the Federal Circuit and its predecessor that "a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of §112 *unless* there is reason to doubt the objective truth of the statements contained therein which much be relied on for enabling support." In re Brana, 34 USPQ2d 1437, 1441 (Fed. Cir. 1995) citing In re Marzocchi, 169 USPQ 367, 369 (CCPA, 1971).

Under 35 U.S.C. § 112, First Paragraph, written description

Claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-55, 65-68, 70-77, and 79-86 are rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matters not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicants respectfully submit that the claimed *in vitro* methods are directed to the use of a group of well known enzymes, *i.e.,* fucosyltransferases, which are adequately described in the specification. In particular, on page 26, lines 1-29, the specification describes various fucosyltransferases useful for the present invention including references that contain the sequence information of these fucosyltransferases. It has also been acknowledged by the Office Action that the sequence information of fucosyltransferases are well known and publicly available. In addition, information is readily available to one skilled in the art with respect to conserved amino acids, binding sites for GDP-fucose, catalytic domain, etc. in the family of fucosyltransferases (See also Exhibit C as discussed above). Therefore, by identifying the enzyme, *i.e.,* fucosyltransferase and by providing structural descriptions, *e.g.,* lacking membrane anchoring domain, the specification clearly describes the fucosyltransferases useful for the claimed methods.

It has long been held by the court that what is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. See MPEP 2163 IIA 3(a) and Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d at 1384, 231 USPQ at 94. If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate written description requirement is met. See, *e.g.,* Vas-Cath, 935 F.2d at 1563, 19 USPQ2d at 1116.

Applicants respectfully submit that, in addition to the detailed descriptions provided by the specification, the examples provided by the specification further demonstrate that the inventors of the present invention were in possession of the claimed invention at the time of filing. Specifically Example 1 describes the use of FucT-VI *in vitro* according to the methods

provided by the present invention to achieve substantially uniform glycosylation pattern for glycopeptides. In Example 2, the inventors demonstrated that while both FucT-VI and FucT-V could be used for *in vitro* glycosylation according to the methods taught by the present invention, FucT-VI was capable of incorporating approximately 8-fold more fucose than FucT-V. Example 3 demonstrate that fucosylation by FucT-VI *in vitro* can be proceed by a sialylation step as taught by the present invention. Therefore, the examples provided by the specification at the time of filing clearly show that the inventors were in possession of the *in vitro* methods, *e.g.*, using fucosyltransferases *in vitro* to achieve substantially uniform glycosylation patterns for glycopeptides.

Furthermore, Applicants respectfully submit that, contrary to the Office Action's assertion that "many structurally unrelated polypeptides are encompassed within the scope of these claims", members of fucosyltransferases used in the present invention are closely related in terms of their structure and function and the specification discloses more than one species of fucosyltransferases useful for the *in vitro* methods. For example, it is known in the art that members of fucosyltransferases share a very high degree of sequence homology with respect to their functional domains. The specification describes more than ten (10) individual members of fucosyltransferase family suitable to be used for the present invention. See page 26, lines 1-29 in the specification. In addition, the examples provided in the specification demonstrate the use of at least two individual fucosyltransferases in the *in vitro* method provided by the present invention. Therefore, the descriptions provided by the specification fully support the scope of the claimed methods.

In summary, the *in vitro* methods using fucosyltransferases for substantially uniform glycosylation is fully described and supported by the specification. Withdrawn of the rejection is respectfully requested.

Under 35 U.S.C. § 103

Claims 1-4, 6, 8, 10-17, 19-21, 65-68, 70-77, and 83-86 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Seed, *et al.*, (PCT Publication No. 96/40881) ("Seed"), or

Kashem et al. (U.S. Pat. No. 5,374,655) ("Kashem"), Natsuka, *et al.*, (U.S. Pat. No. 6,693,183) ("Natsuka"), and Paulson, *et al.*, (PCT Publication No. 98/31826) ("Paulson").

Claims 31-36, 38, 40, 42-49, 51-53, and 79-82 are also rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Seed, *et al.*, (PCT Publication No. 96/40881) ("Seed"), or Kashem et al. (U.S. Pat. No. 5,374,655) ("Kashem"), Natsuka, *et al.*, (U.S. Pat. No. 6,693,183) ("Natsuka"), and Paulson, *et al.*, (PCT Publication No. 98/31826) ("Paulson").

Claims 54-55 are also rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Seed, *et al.*, (PCT Publication No. 96/40881) ("Seed"), or Kashem et al. (U.S. Pat. No. 5,374,655) ("Kashem"), Natsuka, *et al.*, (U.S. Pat. No. 6,693,183) ("Natsuka"), and Paulson, *et al.*, (PCT Publication No. 98/31826) ("Paulson").

Since all three rejections rely on the same prior art references, Applicants will address these rejections together.

The present invention is directed to *in vitro* methods of using fucosyltransferases to achieve substantially uniform fucosylation patterns. Specifically all presently pending claims, except claims 54-55 and certain of their dependent claims, are directed to the use of fucosyltransferases lacking membrane anchoring domain *in vitro*. Claims 54-55 and all of their dependent claims are directed to large scale *in vitro* production of substantially uniform fucosylation patterns using fucosyltransferases. Applicants respectfully submit that none of the cited prior art references teach or suggest the *in vitro* methods provided by the present invention.

Specifically none of the cited prior art references teach or suggest using fucosyltransferases lacking membrane anchoring domains *in vitro*. The Office Action states that "Natsuka et al. teach eukaryotic FTs that lack membrane anchor domain." Applicants respectfully submit that Natsuka does not teach or suggest fucosyltransferases lacking membrane anchoring domains. To the contrary, Natsuka discloses that fucosyltransferases lacking membrane anchoring domain cannot be expressed or obtained using transformed cell lines, thus no fucosylation activity can be detected with fucosyltransferases lacking membrane anchoring domain.

In particular, Natsuka discloses five (5) representative cDNA clones of fucosyltransferase VII, *i.e.* cDNA3, cDNA5, cDNA6, cDNA10, and cDNA14 (Figure 1a, and column 24, lines 45-50). Of these 5 classes of cDNA clones, four of them (cDNA 5, 6, 10, and 14) are trans-

membrane proteins while one of them, *i.e.,* cDNA3 lacks the membrane anchoring domain (column 24, lines 58-67, column 25, lines 1-23). Natsuka discloses that "[i]n contrast to the results obtained with cDNAs 5, 6, 10, and 14, cDNA 3 does not direct detectable sLe^x expression." (See column 26, lines 10-11). Natsuka further discloses that "cells transfected with cDNA3 do not contain any detectable immunoreactive proteins" and this suggests that "the putative initiator codon...in this cDNA does not initiate translation of an immunoreactive product", *i.e.,* does not initiate translation of a fucosyltransferase. In essence, Natsuka discloses that it can not obtain a detectable level of FucT-VII lacking the membrane anchoring domain, *i.e.,* the sequence of FucT-VII does not naturally encode a FucT-VII that lacks the membrane anchoring domain. Such results provided by Natsuka would have cast serious doubts as to whether FucT-VII lacking the membrane anchoring domain is obtainable, and if so, whether it would be useful for fucosylation.

In addition to the teaching away by Natsuka's disclosure with respect to FucT-VII, Costa et al., (Journal of Biological Chemistry, vol. 272, No. 17 pp. 11613-11621, 1997) also disclose that another member of fucosyltransferase family, FucT-III, cannot be used to fucosylate glycopeptides after losing its membrane anchoring domain. (See abstract and discussion sections). The Office Action states that because applicants have excluded FucT-III from certain claims, "it cannot be concluded that what happened with FucT-III would also happen in the case of all other FTs." Applicants respectfully submit that such assertion is without any merit and entirely based on hindsight.

At the time of the present invention, one skilled in the art did not have any knowledge of the present invention. Thus it can not be said that one skilled in the art would have known FucT-III is somewhat unique because of what has been disclosed or claimed in the present invention. In other words, the disclosure of Costa et al., has to be viewed by one skilled in the art without the benefit of the teaching provided by the present invention, *e.g.,* without the benefit of knowing that FucT-III has been excluded from certain claims of the present invention. Therefore, according to the disclosure of Costa et al., one skilled in the art would have had serious doubts as to whether fucosyltransferases lacking the membrane anchoring domain could still maintain their activity in light of the fact that FucT-III lacking the membrane anchoring domain was demonstrated to lose its fucosylation activity.

The Office Action further asserts that irrespective of the disclosure provided by Costa et al., one skilled in the art would have deleted the membrane anchoring domain because of the solubility issue associated with membrane bound proteins. Applicants respectfully submit that such assertion is not supported by any evidence and runs directly against the disclosures of Costa et al., and Natsuka et al., both of which disclose that fucosyltransferases lacking the membrane anchoring domain do not have fucosylation activity. In the absence of any teaching or suggestion, one skilled in the art knowing the disclosure by Costa et al., and Natsuka et al., would have had serious doubts as to whether members of fucosyltransferase family can be used for fucosylation without their membrane anchoring domain. Even if one skilled in the art would have considered deleting the membrane anchoring domain of a fucosyltransferase, other than FucT-III or FucT-VII, he or she would not have had any reasonable expectation of success in light of the fact that both FucT-III and FucT-VII, according to the prior art disclosure, cannot be used for fucosylation without the membrane anchoring domain.

Seed discloses the DNA and amino acid sequences of α(1,3)fucosyltransferase and the use of such full length fucosyltransferase for fucosylation of therapeutic peptides. Seed does not teach or suggest that such fucosyltransferase can be used without its membrane anchoring domain for fucosylation of glycopeptides.

Kashem discloses the use of fucosyltransferase for a single fucosylation site on a simple chemical compound, *i.e.,* oligosaccharides. Kashem does not teach or suggest that fucosyltransferases can be used for any fucosylation of chemical compounds without their membrane anchoring domain.

Paulson discloses the use of sialyltransferases for *in vitro* sialylation of glycoproteins. Paulson does not teach or suggest that fucosyltransferases can be used without their membrane anchoring domain for the purpose of fucosylation of glycopeptides. As acknowledged by the Office Action, Paulson is cited to show glycosylation of peptides using enzymes other than fucosyltransferases which is in response to claim limitations such as glycosylation by an enzyme other than an fucosyltransferase.

Therefore, Seed, Kashem, and Paulson can not cure the deficiency of Natsuka.

In summary, none of the cited prior art references teach or suggest the use of fucosyltransferases without their membrane anchoring domain and it would not have been

obvious to one skilled in the art that fucosyltransferases without their membrane anchoring domain could be used *in vitro*, especially in light of substantial "teaching away" disclosures in the field. Thus the *in vitro* methods of using fucosyltransferases without their membrane anchoring domain as provided by the present invention are not obvious over the cited prior art.

With respect to claims 54-55 and newly added dependent claims thereof, applicants respectfully submit that these claims are directed to *in vitro* large scale production of substantially uniform fucosylation patterns of glycopeptides and none of the cited prior art references teach or suggest any commercial production *in vitro*, especially using at least about 500 mg of glycopeptide as recited in the claims of the present invention. The Office Action acknowledges that none of the references disclose explicitly a large scale fucosylation process *in vitro*. Nevertheless the Office Action speculates that the methods disclosed by Seed and Kashem can be easily scaled up for large scale commercial production of substantially uniform fucosylation patterns *in vitro*. The Office Action fails to provide any evidence to support such speculation, therefore the Office Action fails to make its *prima facie* case of obviousness against the present invention.

Furthermore, applicants respectfully submit evidence of secondary considerations in support of non-obviousness of the present invention including claims 54-55 and their dependent claims.

As described in the Declaration by David Zopf, a copy of which is attached as Exhibit D, the *in vitro* methods provided by the present invention had encountered initial expert skepticism and subsequently enjoyed great commercial success, *e.g.*, licensing by others and collaboration with others by contributing the *in vitro* methods of the present invention. Specifically, prior to the filing of the present invention expert in the field expressed serious doubts and skepticism with respect to large scale production using the *in vitro* fucosylation methods provided by the present invention. As a representative example, we provide a letter from professor Dr. James E. Bailey at Institute of Biotechnology in Zurich, Switzerland (Exhibit E), which voiced strongly that large scale glycosylation should be conducted *in vivo,* rather than *in vitro* using methods provided by the present invention.

Professor Bailey had stated in his letter that "process complications and costs associated with producing and utilizing a glycosyltransferase and also supplying the donor substrate make

the exogenous manipulation of glycosylation generally far less attractive than engineering the cells to maximize the production of the desired glycoform." Professor Bailey further expressed his doubts about *in vitro* methods of the present invention by stating that "only one particular glycoform would be a suitable substrate for arriving at the desired final product, greatly reducing the potential yield of such exogenous glycosylation manipulation." Professor Bailey concluded that "[f]ailure of much simpler cofactor-requiring enzyme catalyzed reactions to gain industrial success in competition with whole-cell biocatalysis speaks very strongly in my opinion against the competitive prospects of *in vitro* remodeling of glycosylation."

Therefore, professor Bailey's letter clearly shows, as an example, expert skepticism towards using fucosyltransferases *in vitro*, especially for large scale production of substantially uniform fucosylation patterns prior to the filing of the present invention.

Subsequent to overcoming expert skepticism, the present invention has enjoyed great commercial success. Many companies have collaborated with Neose Technologies (current owner of the invention) including licensing the *in vitro* methods of the present invention for large scale fucosylation production of therapeutic peptides. Dr. Zopf has provided two representative examples in his Declaration to illustrate such success in detail. As indicated in the Declaration, the present invention has been licensed by Wyeth/Ayerst Laboratories and Avant Immunotherapeutics, in both cases the *in vitro* methods provided by the present invention successfully delivered substantially uniform fucosylation patterns for desired therapeutic glycoproteins on a large commercial scale. As further indicated in Dr. Zopf's Declaration, projects with many other collaboration partners have also successfully utilized the *in vitro* methods provided by the present invention, especially in large scale commercial production.

The great commercial value of the present invention agreed upon by others in the industry undeniably testifies for the great success of the *in vitro* fucosylation methods provided by the present invention. In addition, such commercial value and success also demonstrate failure by others in the industry and that the *in vitro* fucosylation methods of the present invention provides long felt needs in the commercial world.

In summary, the Office Action fails to provide any evidence to show why the methods disclosed in the cited prior art can be easily scaled up for large scale production. Therefore the Office Action fails to make its *prima facie* obviousness case. Furthermore, applicants provide

ample evidence of secondary considerations supporting the non-obviousness of the *in vitro* fucosylation methods provided by the present invention over the disclosures of the prior art, especially in light of expert skepticism encountered early on and the commercial success subsequently enjoyed by the present invention.

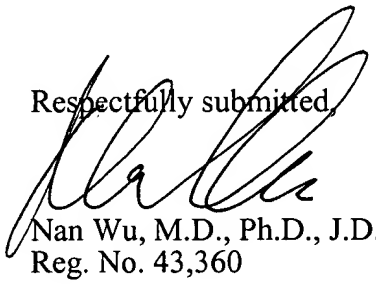
In conclusion, the *in vitro* fucosylation methods provided by the present invention is not obvious over the disclosures of the cited prior art. Applicants respectfully request withdrawal of the rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

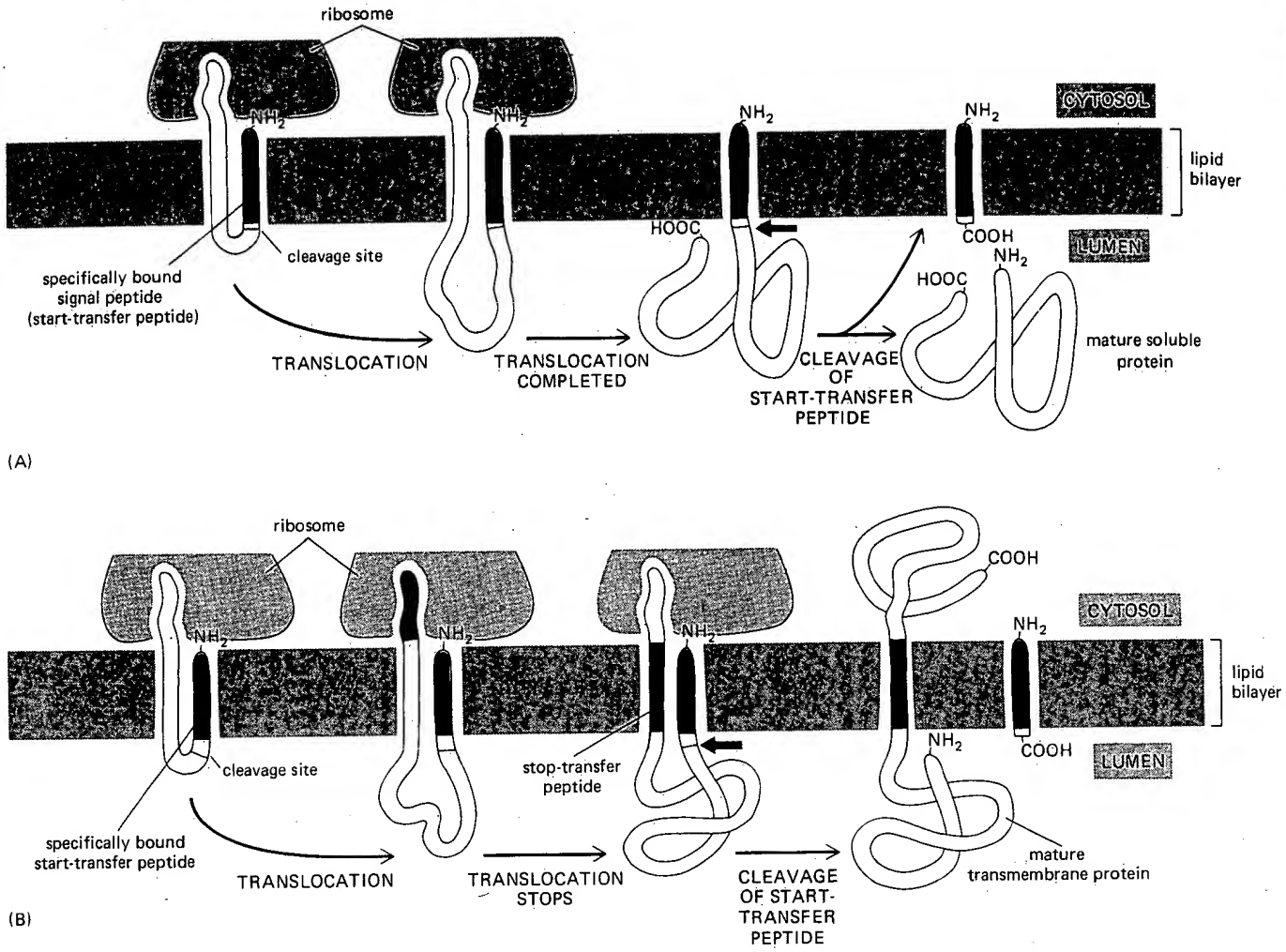
Applicants respectfully request a telephone interview if the Examiner believes that the claims as amended are not in condition for allowance in light of the response submitted above. The undersigned can be reached at 415-442-1000.

Respectfully submitted,



Nan Wu, M.D., Ph.D., J.D.
Reg. No. 43,360

MORGAN, LEWIS & BOCKIUS, LLP
One Market, Spear Street Tower
San Francisco, California 94105
Tel: 415-442-1000
Fax: 415-442-1001
Attachments



p. 293). It is thought that an internal signal peptide serves as a start-transfer signal in these proteins and initiates translocation, with each translocation event proceeding until the next stop-transfer peptide is reached. Thus the fundamental unit translocated is a loop of polypeptide between two hydrophobic segments (one start-transfer peptide and one stop-transfer peptide), with both of these peptides serving as α-helical membrane-spanning domains in the mature protein. A possible mechanism that might insert one such loop into the membrane is illustrated in Figure 8–46. In complex multipass transmembrane proteins, in which many hydrophobic α helices span the bilayer, a second start-transfer peptide would reinitiate translocation farther down the chain until the next stop-transfer peptide halted it once again, and so on for subsequent start-transfer and stop-transfer peptides (see Figure 8–48D).

Figure 8–45 The topology of protein translocation across the ER membrane is illustrated for two simple cases. The translocational intermediate is thought to contain a loop of polypeptide chain, in which the signal peptide (also called the start-transfer peptide) forms half of the stem of the loop and the region of the polypeptide being translocated across the membrane at any instant forms the other half. When there is one start-transfer peptide and no stop-transfer peptide, the entire polypeptide is translocated across the membrane, and cleavage of the start-transfer peptide releases the mature protein into the lumen of the ER as a soluble protein (A). When one start-transfer peptide and one stop-transfer peptide are present, translocation stops when the stop peptide enters the stem of the loop while protein synthesis continues on the cytosolic side of the membrane; after cleavage of the start-transfer peptide, the mature protein is left spanning the lipid bilayer of the ER with one domain protruding on each side (B).

8-28 The General Conformation of a Transmembrane Protein Can Often Be Predicted from the Distribution of Its Hydrophobic Amino Acids[40]

Although stop-transfer peptides are generally more hydrophobic than start-transfer peptides, they can sometimes act as start-transfer peptides if their location in a protein is changed. Thus the distinction between hydrophobic start-transfer and stop-transfer peptides results in part from the order in which they occur in the nascent polypeptide chain. It seems that the translocation machinery in the ER membrane begins scanning an unfolded polypeptide chain for hydrophobic segments at its amino terminus and proceeds toward the carboxyl terminus—in the same direction as the protein is synthesized. The SRP recognizes the first appropriate segment and thereby sets the "reading frame." The next appropriate hy-

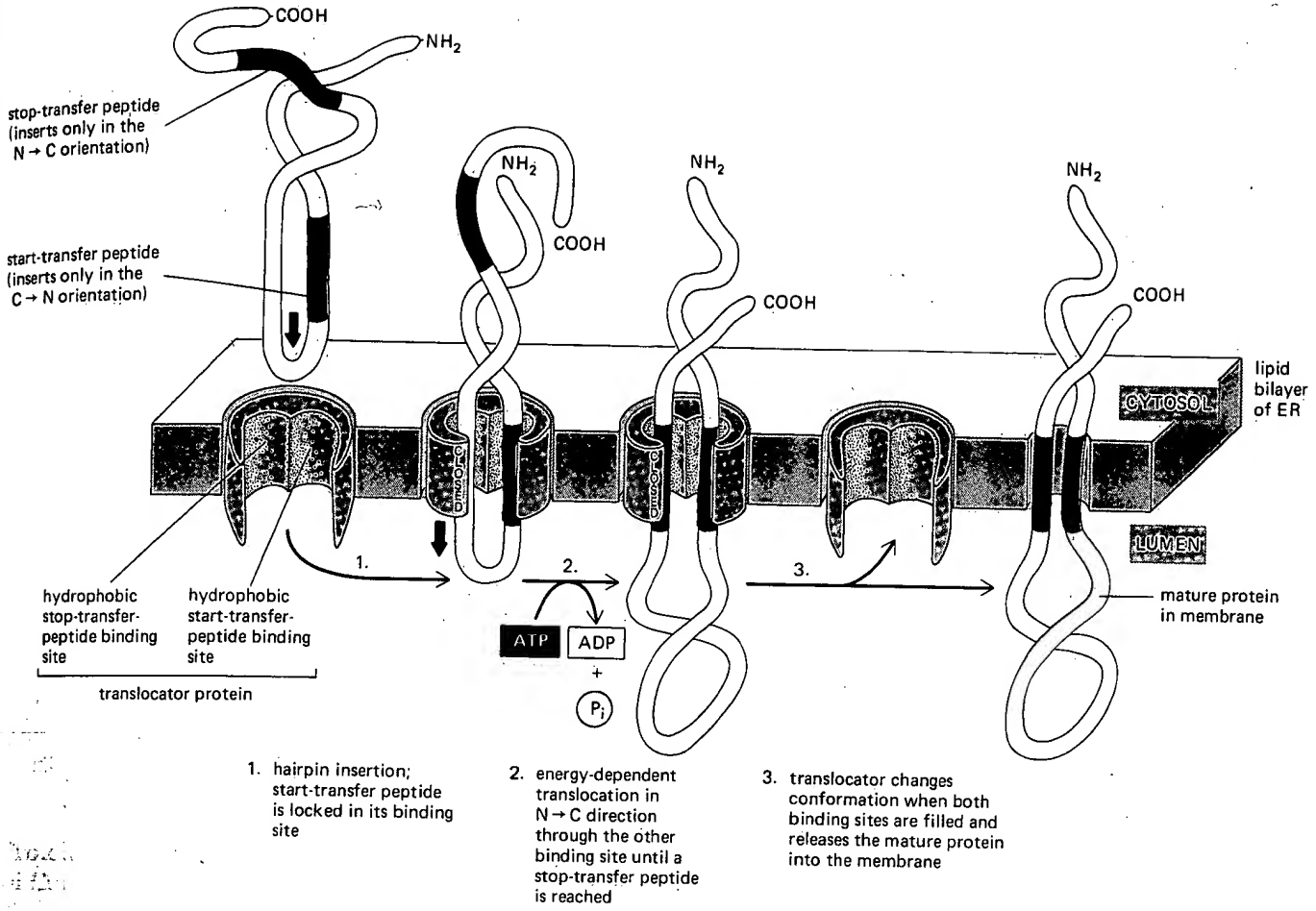


Figure 8–46 A hypothetical model for the insertion of an internal loop of polypeptide chain into the lipid bilayer of the ER. A protein translocator is postulated to have two conformations, "closed" and "open." On binding the start-transfer peptide of a protein to be translocated, the translocator enters the closed state and becomes active in translocation. But it flips back to an inactive, open conformation and discharges its protein as soon as a stop-transfer peptide enters its other binding site.

drophobic segment is recognized as a stop-transfer peptide, causing the region of the polypeptide chain in between to be threaded through the membrane (see Figure 8–46). It has been postulated that a similar process continues until all of the hydrophobic regions in the protein are inserted into the membrane.

This mechanism for membrane insertion means that one can often predict the topography of a membrane protein from its amino acid sequence. One begins by scanning for contiguous regions of about 20–30 amino acid residues with a high degree of hydrophobicity. These segments are long enough to span a membrane as an α helix, and they can often be identified by means of a *hydropathy plot* (Figure 8–47). The topology of the polypeptide chain can then be predicted on the assumption that the first segment, scanning from the amino terminus, has a start-transfer function and that stop-transfer peptides thereafter alternate with further start-transfer peptides. Four examples are illustrated in Figure 8–48.

Because membrane proteins are always inserted from the cytosolic side of the ER in this programmed manner, all copies of the same polypeptide chain will have the same overall orientation in the bilayer. This generates an asymmetrical ER membrane in which the protein domains exposed on one side are different from those domains exposed on the other. This asymmetry is maintained during the many membrane budding and fusion events that transport the proteins made in the ER to other cell membranes (see Figure 8–10), and it therefore determines the orientation of the proteins in these membranes as well.

When proteins are dissociated from a membrane and reconstituted into artificial lipid vesicles (see p. 287), a random mixture of right-side-out and inside-out protein orientations usually results. Thus the protein asymmetry observed in cell membranes is thought to result solely from the process by which proteins are inserted into the ER membrane from the cytosol.

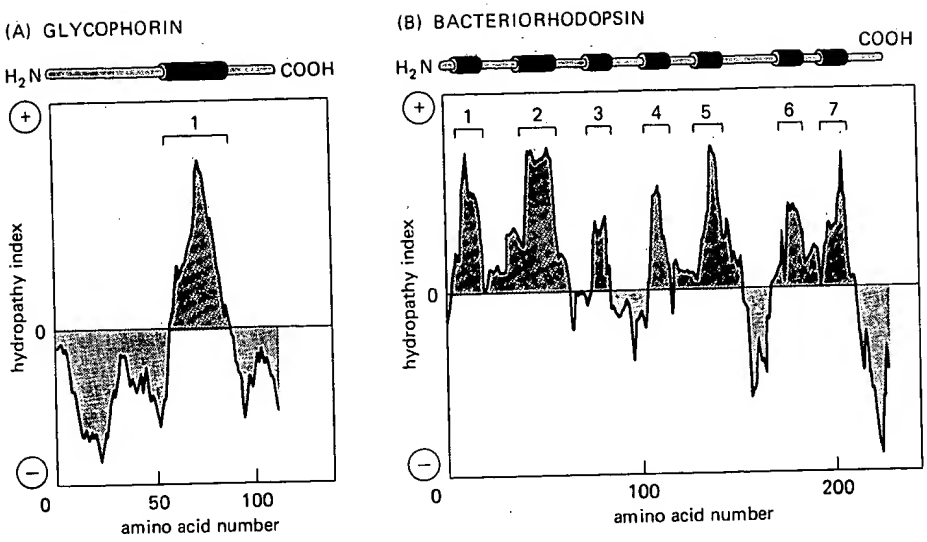
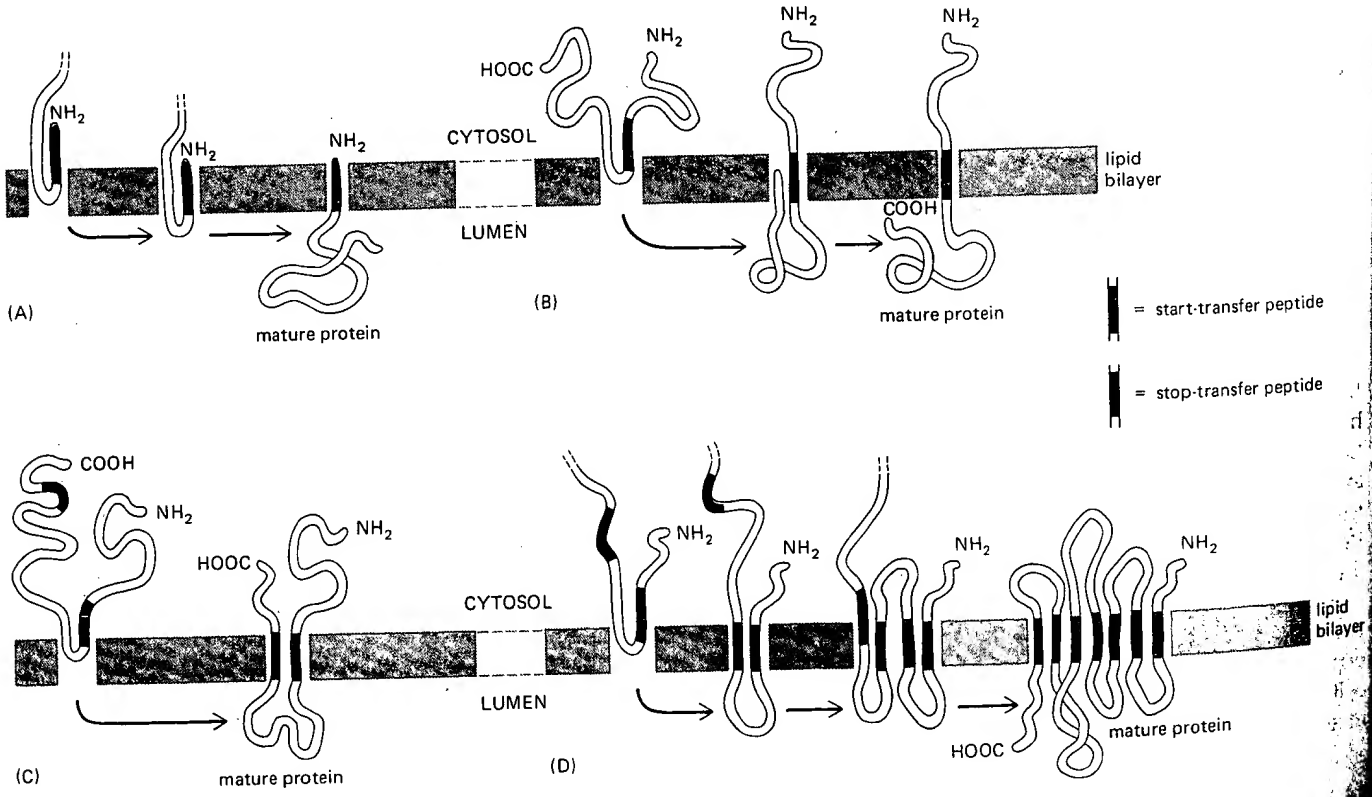


Figure 8–47 Localization of potential hydrophobic (membrane-spanning) segments in a polypeptide chain through the use of hydropathy plots. The free energy needed to transfer successive segments of a polypeptide chain from a nonpolar solvent to water is calculated from the amino acid composition of each segment using data on model compounds. This calculation is made for segments of a fixed size (usually around 10 amino acid residues), beginning with each successive amino acid in the chain. The "hydropathy index" of the segment is plotted on the *Y* axis as a function of its location in the chain. A positive value indicates that free energy is required for transfer to water (that is, the segment is hydrophobic), and the value assigned is an index of the amount of energy needed. Peaks in the hydropathy index appear at the positions of hydrophobic segments in the amino acid sequence. Two examples are shown: (A) glycophorin (see p. 291) has a single membrane-spanning hydrophobic domain and one corresponding peak in the hydropathy plot; (B) bacteriorhodopsin (see p. 293) has seven membrane-spanning helices and seven corresponding peaks in the hydropathy plot. (Adapted from D. Eisenberg, *Annu. Rev. Biochem.* 53:595–624, 1984.)

Figure 8–48 The topology of a membrane protein is dictated by alternating stop-transfer and signal (start-transfer) peptides. In all the examples shown, signal peptides are not cleaved. The hypothetical protein translocator is assumed to function in the manner illustrated previously in Figure 8–46. (A) When an amino-terminal signal peptide is not cleaved and no stop-transfer peptide is present; a membrane protein with a single carboxyl-terminal domain that protrudes on the luminal side of the ER membrane is generated. (B) When the signal peptide is internal, a membrane protein with an amino-terminal cytoplasmic domain and a carboxyl-terminal luminal domain is produced. (C) When a stop-transfer peptide follows an internal signal peptide, three separate domains will protrude from a membrane protein. (D) Membrane proteins that span the bilayer many times can be generated by a simple extension of the same principles, employing alternating signal peptides and stop-transfer peptides.



Glycobiology vol. 14 no. 10 pp. 883–893, 2004
doi:10.1093/glycob/cwh112
Advance Access publication on June 9, 2004

Production of a complement inhibitor possessing sialyl Lewis X moieties by *in vitro* glycosylation technology

Lawrence J. Thomas[1,3], Krishnasamy Panneerselvam[1,4], David T. Beattie[3], Michele D. Picard[3], Bi Xu[3], Charles W. Rittershaus[3], Henry C. Marsh Jr.[3], Russell A. Hammond[3], Jun Qian[4], Tom Stevenson[4], David Zopf[4], and Robert J. Bayer[2,4]

[3]AVANT Immunotherapeutics, 119 Fourth Ave., Needham, MA 02494, and [4]Neose Technologies, 102 Witmer Road, Horsham, PA 19044

Received on March 24, 2004; revised on June 4, 2004; accepted on June 4, 2004

Recombinant soluble human complement receptor type 1 (sCR1) is a highly glycosylated glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. sCR1-sLe^x produced in the FT-VI-expressing mutant CHO cell line LEC11 exists as a heterogeneous mixture of glycoforms, a fraction of which include structures with one or more antennae terminated by the sialyl Lewis X (sLe^x) [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc] epitope. Such multivalent presentation of sLe^x was shown previously to effectively target sCR1 to activated endothelial cells expressing E-selectin. Here, we describe the use of the soluble, recombinant α2-3 sialyltransferase ST3Gal-III and the α1-3 fucosyltransferase FT-VI *in vitro* to introduce sLe^x moieties onto the N-glycan chains of sCR1 overexpressed in standard CHO cell lines. The product (sCR1-S/F) of these *in vitro* enzymatic glycan remodeling reactions performed at the 10-g scale has approximately 14 N-glycan chains per sCR1 molecule, comprised of biantennary (90%), triantennary (8.5%), and tetraantennary (1.5%) structures, nearly all of whose antennae terminate with sLe^x moieties. sCR1-S/F retained complement inhibitory activity and, in comparison with sCR1- sLe^x produced in the LEC11 cell line, contained twice the number of sLe^x moieties per mole glycoprotein, exhibited a twofold increase in area under the intravenous clearance curve in a rat pharmacokinetic model, and exhibited a 10-fold increase in affinity for E-selectin in an *in vitro* binding assay. These results demonstrate that *in vitro* glycosylation of the sCR1 drug product reduces heterogeneity of the glycan profile, improves pharmacokinetics, and enhances carbohydrate-mediated binding to E-selectin.

Key words: glycoengineering/glycoprotein remodeling/glycosylation/glycosyltransferase

Introduction

Soluble complement receptor type 1 (sCR1) is a recombinant glycoprotein that has been shown to inhibit the progression of the complement cascade in both the classical and alternative pathways by inhibiting the stable formation of C3 and C5 convertases and by serving as a cofactor in the proteolytic degradation of C3b and C4b by Factor I (Weisman *et al.*, 1990). The administration of sCR1 has been shown to be effective in a number of animal disease models of human complement–dependent ischemia-reperfusion injury for tissues, such as heart (Lazar *et al.*, 1999), liver (Lehmann *et al.*, 1998), hind limb (Kyriakides *et al.*, 2001a), lung (Naka *et al.*, 1997), and intestine (Williams *et al.*, 1999). Complement inhibition by sCR1 has been shown to reduce hyperacute rejection (Pruitt *et al.*, 1997) and enhance graft survival in many established transplant models (Kallio *et al.*, 2000; Pratt *et al.*, 1996; Stammberger *et al.*, 2000).

In some clinical situations, complement inhibition therapy could be more effective if it were targeted directly to sites of endothelial activation. At sites of inflammation, activated endothelial cells express E-selectin and P-selectin, surface adhesins with carbohydrate-binding domains that recognize the carbohydrate epitope, sLe^x (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) (Lasky, 1995).

Previously we have described sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), a variant of the sCR1 glycoprotein conveniently produced in LEC11 cells transfected with the sCR1 gene. LEC11 is a mutant Chinese hamster ovary (CHO) cell line that expresses fucosyltransferase VI (FT-VI), a Golgi enzyme capable of adding fucose in α1-3 linkage to GlcNAc in oligosaccharide chains that terminate with either Galβ1-4GlcNAcβ1 ... or Neu5Acα2-3Galβ1-4GlcNAcβ1 ... (Zhang *et al.*, 1999). Of the 25 potential N-glycosylation sites within the sCR1 polypeptide sequence, 13–15 are occupied, the majority with biantennary chains, creating the possibility for as many as 30 sLe^x moieties per molecule of sCR1-sLe^x. However, a previously reported analysis of the N-glycans of sCR1-sLe^x showed heterologous oligosaccharides with a variety of partially sialylated and fucosylated structures yielding less than the maximal number of sLe^x moieties (Picard *et al.*, 2000; Rittershaus *et al.*, 1999). Similar heterogeneity of glycans in CHO-expressed glycoproteins has been described previously and attributed to incomplete Golgi processing, post-secretion degradation due to glycohydrolases released into cell culture media, or both (Goochee *et al.*, 1991; Jenkins *et al.*, 1996).

In this article we describe a process to introduce sLe^x moieties onto the N-glycan chains of sCR1 produced in standard CHO cell lines using *in vitro* enzymatic synthesis.

[1]These authors contributed equally to this work.
[2]To whom correspondence should be addressed; e-mail: bbayer@neose.com

This method employs serial treatment of sCR1 with soluble recombinant rat ST3Gal-III and human FT-VI to give an sCR1-sLe^x product, designated sCR1-S/F (for differentiation from the LEC11 product) in which the antennae of N-glycans are nearly uniformly terminated with sLe^x epitopes. The benefits of *in vitro* glycan remodeling include improved pharmacokinetics, enhanced binding to E-selectin, and a means to improve product homogeneity. Enzymatic remodeling is demonstrated at the 10-g scale.

Results

In vitro *remodeling of sCR1 glycans*

sCR1 (250 mg) expressed in CHO cells was sialyated by treatment with ST3Gal-III plus CMP-sialic acid to give a product designated sCR1-S. After an aliquot was removed from the reaction mixture for analysis, the remaining sCR1-S was fucosylated in the same reaction vessel by the addition of FT-VI plus GDP-fucose to give a product designated sCR1-S/F. After purification by serial chromatography on ceramic hydroxyapatite and Q Sepharose, the reaction products had the same retention time and percent purity (98.5%) by reversed phase high-pressure liquid chromatography (RP-HPLC) as the starting material, sCR1 (data not shown). Chemical and functional properties of these molecules were compared with those of sCR1-sLe^x, a molecule previously produced in the FT-VI-expressing LEC11 CHO cell line and shown to contain some N-linked biantennary glycans terminated with the sLe^x tetrasaccharide (Picard *et al.*, 2000).

From the mannose content of sCR1, sCR1-S, sCR1-S/F, and sCR1-sLe^x (Table I) it may be inferred that these molecules contain ~13–15 N-glycan chains per mol protein (assuming 3 mol mannose per N-glycan chain). The fluorophore-assisted carbohydrate electrophoresis (FACE) oligosaccharide profile for sCR1 (Figure 1) shows three major bands consistent with a biantennary structure containing zero, one, or two sialic acid residues, as described previously (Picard *et al.*, 2000). The monosaccharide composition of sCR1 (Table I) suggests that ~57% of total galactosyl residues are substituted with sialic acid (19 mol sialic acid/ 33.2 mol galactose). By comparison, the FACE oligosaccharide profile for sCR1-S (Figure 1) shows one major band that migrates at a position consistent with a biantennary structure containing two sialic acid residues, and monosaccharide analysis reveals the galactose/sialic acid ratio to be 1:1 (Table I).

FACE analysis of glycans from sCR1-S/F, prepared by enzymatic fucosylation of sCR-S, suggests that N-glycans are predominantly biantennary and that fucosylation at both antennae is nearly complete (Figure 1). The dominant oligosaccharide band derived from sCR1-S/F was cut out and extracted from the gel. Sequential removal of monosaccharide residues from the extracted glycoprotein using specific glycosidases gave products with mobilities consistent with α1-6 core-fucosylated, biantennary N-glycans (Figure 2). Monosaccharide analysis of sCR1-S/F shows the presence of 39.3 moles fucose per mol sCR1-S/F, a figure in agreement with the prediction from theory that 39–45 fucose residues per mol protein would be present if all N-glycans were core fucosylated and enzymatic fucosylation of antennary GlcNAc residues were complete.

The FACE oligosaccharide profile for sCR1-sLe^x, a glycoprotein produced in LEC11 CHO cells, shows at least seven bands (Figure 1) with some common to sCR1 and others shown previously (Picard *et al.*, 2000) to represent core fucosylated structures with α1-3 fucosylation at one or more antennae. Heterogeneity in the degree of fucosylation of the N-glycan chains from sCR1-sLe^x also can be appreciated from the results of monosaccharide analysis (Table I). For example, it may be calculated (assuming 3 mannose residues per chain) that sCR1-sLe^x contains an average of 2.5 fucosyl residues per glycan chain. By contrast, the fucose content per glycan chain increases from 0.95 for

Table I. Monosaccharide Content (mol/mol glycoprotein) by HPLC analysis

	sCR1	sCR1-S	sCR1-S/F	sCR1-sLe^x
Glucosamine	62	48	48	62
Galactose	33	28	27	38
Mannose	44	39	35	40
Fucose	16	12	39	33
Sialic acid	19	30	28	27
Sialic acid/galactose	0.57	1.09	1.06	0.70
Glycosylation sites/sCR1	15	13	12	13
Estimated sLe^x/sCR1-sLe^x*	n.a.	n.a.	28	14

*Estimated sLe^x/sCR1-sLe^x = (Fuc/sCR1-sLe^x − sites/sCR1-sLe^x) × Sial/Gal ratio.

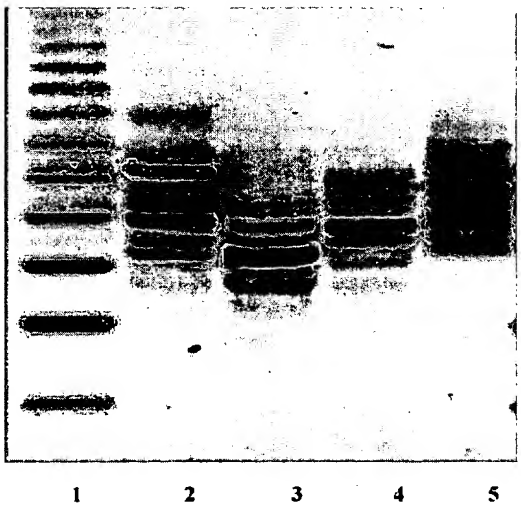


Fig. 1. FACE profiling of oligosaccharides from sCR1-sLe^x and sCR1 before and after enzymatic remodeling: (1) Glyko oligosaccharide standard ladder, (2) sCR1, (3) sCR1-S, (4) sCR1-S/F, (5) sCR1-sLe^x. The oligosaccharide profile of sCR1 (lane 2) contains predominantly bands representing biantennary structures with two sialic acids (bottom band), one sialic acid (middle band), and no sialic acids (top band).

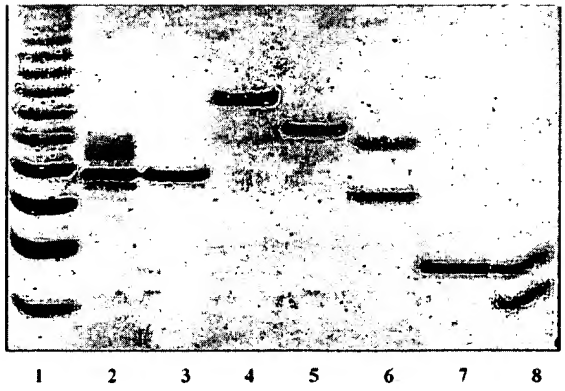


Fig. 2. FACE analysis of oligosaccharides from sCR1-S/F after serial treatment with glycosidases. The dominant oligosaccharide band derived from sCR1-S/F (lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was digested sequentially to remove each monosaccharide residue starting at the terminal sialic acid residue and ending at the trimannosyl core: (1) Glyko oligosaccharide standard ladder; (2) total N-linked oligosaccharides of sCR1-S/F; (3) purified dominant band (band 1) from lane 2; (4) band 1 treated with NANaseIII (cleaves α2-3, 4, 6, 8, and 9 linked sialic acid); (5) band 1 treated with NANaseIII and FucaseIII (cleaves α1-3 and 4 fucose); (6) band 1 treated with NANaseIII, FucaseIII, and GalaseIII (cleaves terminal galactose); (7) band 1 treated with NANaseIII and FucaseIII, GalaseIII, and hexosaminidase; (8) standard trimannosyl core N-glycans with (upper band) and without α1-6 fucose.

Fig. 3. FACE analysis of oligosaccharides from sCR1-S/F treated with sialidases. The dominant oligosaccharide band (band 1) derived from sCR1-S/F (see Figure 2, lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was subjected to enzymatic digestion to remove terminal sialic acid: (1) Glyko oligosaccharide standard ladder; (2) band 1 from sCR1-S/F; (3) band 1 treated with NANasel (cleaves α2-3 linked sialic acid); (4) band 1 treated with NANaseIII (cleaves α-3, 4, 6, 8, and 9 linked sialic acid).

sCR1-S to 3.3 for sCR1-S/F, a result that correlates well with the single band visualized by FACE analysis of sCR1-S/F (Figure 1).

Oligosaccharide sequencing using FACE

The linkage of terminal sialic acids on sCR1-S/F was assessed by digestion with specific neuraminidases (Figure 3). Complete removal of sialic acid by treatment of band 1 from sCR1-S/F with NANase I indicates that sialic acid residues are α2-3 linked, as expected.

Optimization of sialylation reaction for scale-up

To establish conditions for scaleup of sialylation, sCR1 (5 mg/ml) was incubated with varying amounts of ST3Gal-III (10, 25, 75, 100, 200, 300 and 400 U/ml) and 5 mM CMP–sialic acid plus a trace amount of radiolabeled CMP–sialic acid for 24 h at 32°C. At an ST3Gal-III concentration of 150 mU/ml, incorporation of radiolabeled sialic acid reached 91% of maximum after 24 h and 100% at 48 h. The lowest concentration of enzyme required to give nearly maximum incorporation (~40 mol sialic acid/mol protein) under these conditions was 25 mU/ml ST3Gal-III (Figure 4). It should be noted that the contribution of triantennary and tetraantennary species may be responsible for the observation that more than 30 moles of sialic acid was added per mole of sCR1. Increasing the CMP–sialic acid concentration from 5 mM to 10 mM did not affect the level of sialylation of sCR1 at any of the ST3Gal-III concentrations tested (data not shown). HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of glycans

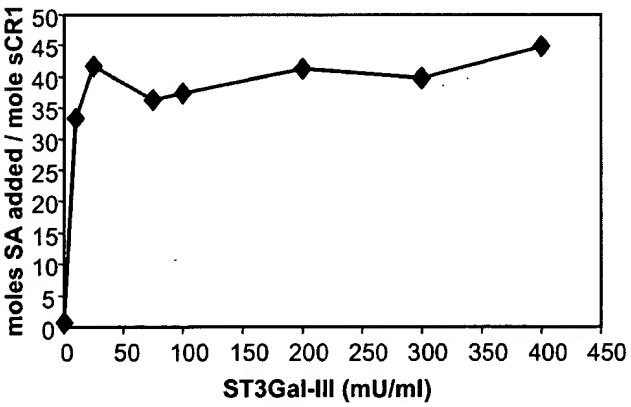


Fig. 4. Incorporation of sialic acid into sCR1 at increasing concentrations of ST3Gal-III in a 24-h reaction. The moles of sialic acid added are estimated from incorporation of radiolabeled CMP–sialic acid. Incorporated radiolabel is separated from free by gel filtration on a $TSKG2000_{SWXL}$ column.

released from sCR1-S revealed that at all concentrations of enzyme tested, the product contained predominantly disialylated, biantennary, core fucosylated N-glycans (data not shown). A concentration of 200 mU ST3Gal-III/ml was chosen for scale-up to ensure completeness of reaction.

Optimization of fucosylation

To establish conditions for scale-up of fucosylation, sCR1-S (5 mg/ml) was incubated with varying amounts of

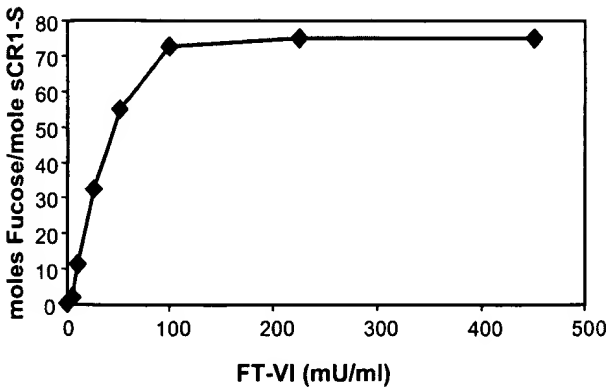


Fig. 5. Incorporation of fucose into sCR1-S at increasing concentrations of FT-VI in a 24-h reaction. The moles of fucose added are estimated from incorporation of radiolabeled GDP-fucose. Incorporated radiolabel is separated from free by gel filtration on a $TSKG2000_{SWXL}$ column.

FT-VI (10, 20, 40, 60, 100, 220, 440 mU/ml) and 5 mM GDP-fucose plus a trace of radiolabeled GDP-fucose for 24 h at 32°C. The lowest concentration of enzyme required to give nearly maximum incorporation of fucose under these conditions was 100 mU/ml FT-VI (Figure 5). Increasing the GDP-fucose concentration from 5 mM to 10 mM did not increase fucose incorporation at several different FT-VI concentrations tested (data not shown).

For products of reactions run at all concentrations of FT-VI $\geq$ 100 mU/ml, the glycan structures identified by HPLC and MALDI-TOF MS were almost the same and essentially indistinguishable from the structures described next for sCR1-S/F produced at the 10-g scale.

Remodeling at 10-g scale

Purified sCR1 (10 g in a volume of 2 L) was incubated first with ST3Gal-III plus CMP–sialic acid at 32°C for 36 h and then, following addition of FT-VI plus GDP-fucose, incubated at 32°C for another 36-h period..

FACE analyses of glycans from sCR1, sCR1-S, and sCR1-S/F for reactions performed at the 10-g scale (data not shown) were essentially indistinguishable from FACE results obtained at the 250-mg scale (Figure 1), suggesting that occupancy of potential acceptor sites for ST3Gal-III and FT-VI on sCR1 at the 10-g scale was nearly complete.

HPLC profiles for 2-AA-derivatized glycans of sCR1, sCR1-S, and sCR1-S/F are shown in Figure 6 and the percentages of glycan species estimated from integrated peak areas are summarized in Table II. After *in vitro* sialylation with ST3Gal-III, neutral glycans, comprising 50% of carbohydrate chains in sCR1, are reduced to 2% of chains in sCR1-S, and monosialo-glycans likewise decrease to from 35% in sCR1 to 17.5% in sCR1-S (Figure 6 and Table II). Overall, about 90% of N-glycans are biantennary and these chains contain an average of 1.8 sialic acid moieties per glycan. Among the minority of biantennary glycans on sCR1-S that are monosialylated, some lack galactose on one antenna, whereas others contain two galactosyl residues, only one of which is sialylated. The remaining 10% of glycans are fully sialylated triantennary (8.5%) or tetra-antennary (1.5%) structures.

After fucosylation of sCR1-S to create sCR1-S/F, HPLC and MALDI-TOF MS analyses (Table III and Figure 7)

Table II. HPLC data summary of large scale remodeling

Glycan species	Native protein (sCR1) (%)	Sialylated protein (sCR1-S) (%)	Sialylated and fucosylated protein (sCR1-S/F) (%)
Neutral	50.5	2.0	4.0
1 charge	35.0	17.5	25.5
2 charges	13.0	70.5	68.5
3 charges	1.5	8.5	1.5
4 charges	ND*	1.5	0.5

*Not detected.

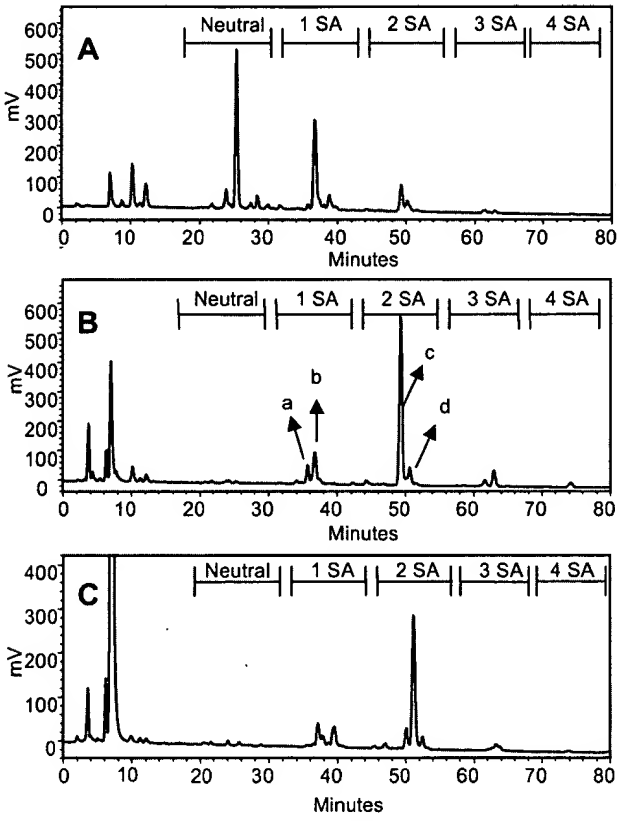
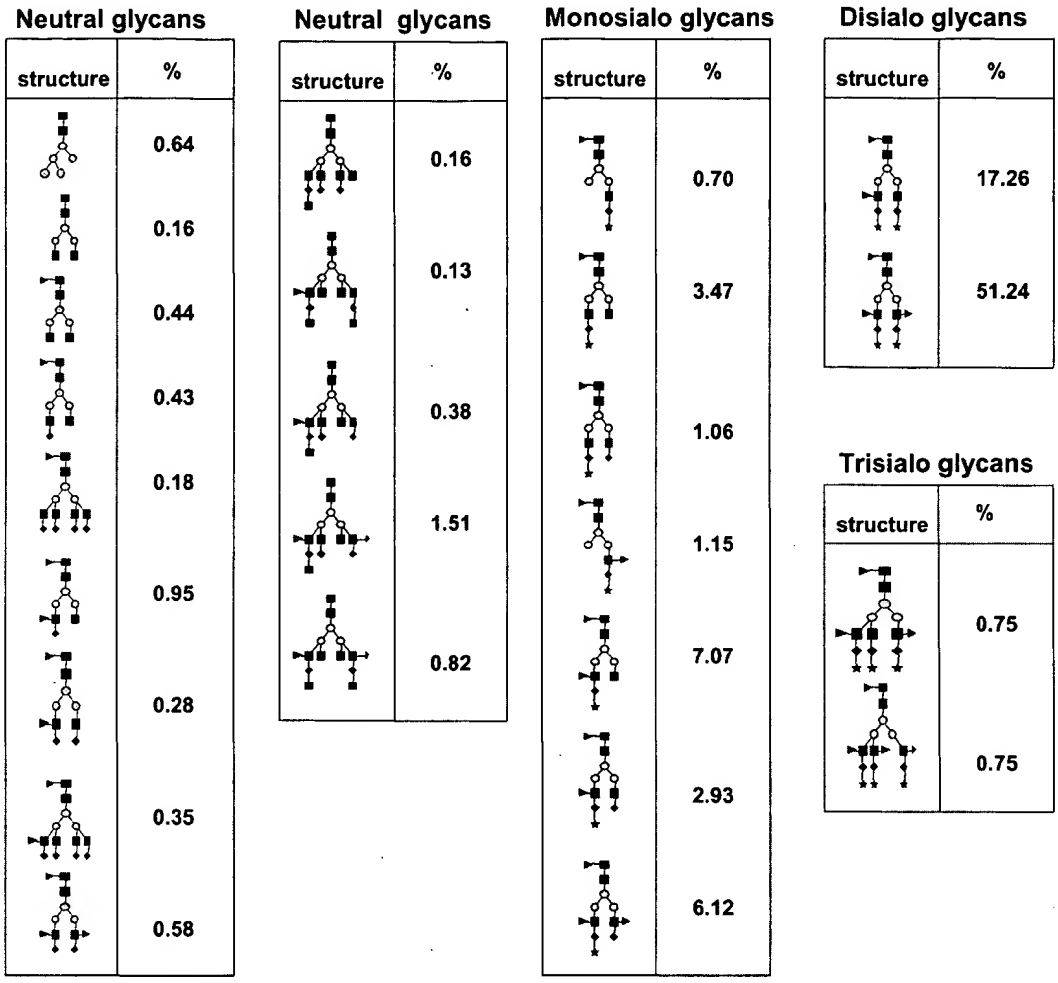


Fig. 6. RP-HPLC analysis of 2-AA-oligosaccharides before and after enzymatic remodeling at the 10-g scale: (A) sCR-1; (B) sCR1-S; (C) sCR1-S/F. MALDI-TOF MS analysis (data not shown) of 2-AA-oligosaccharides from sCR1-S (B) indicated that: peak a contains monosialylated biantennary glycans that lack terminal galactose on one antenna; peak b, constituting 12% of biantennary glycans, contains biantennary glycans with two galactose residues, but only one sialic acid; peaks c and d contain disialylated, biantennary glycans, with and without core fucose, respectively.

Table III. sCR1-S/F glycans



Blue squares represent N-acetylglucosamine, yellow circles represent mannose, green triangles represent fucose, red diamonds represent galactose, and asterisks represent sialic acid.

showed that more than 95% of the glycans were fucosylated by FT-VI. About 62% of the total N-glycans gained two fucose residues, and ~30% gained a single fucose residue. Failure to accept two fucosyl residues was in part due to missing galactosyl residues on one or more antennae. From these results it can be estimated that the sCR1-S/F molecules created by consecutive *in vitro* sialylation and fucosylation reactions contain, on average, 28 sLe^x epitopes per protein molecule, whereas sCR1-sLe^x, glycosylated and secreted by the FT-VI-expressing LEC11 CHO cell, contains ~14 sLe^x epitopes per protein molecule (Table I).

To check the stability of sCR1 under conditions of incubation with glycosyltransferases, a small amount of protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after each remodeling reaction. There was no evidence of degradation of the polypeptide after incubation with either ST3Gal-III or FT-VI (data not shown).

Pharmacokinetics

When sCR1-S prepared at the 250-mg scale was injected intravenously into rats, the observed area under the curve (AUC_{last}) was twofold greater than the AUC_{last} for sCR1 ($p < 0.004$), indicating a significantly greater exposure of the more completely sialylated form of the complement inhibitor to intravascular cells following dosing (Figure 8).

In vitro *antihemolytic activity*

The IH_{50} values for sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F as inhibitors of human complement–mediated lysis of sheep red blood cells were found to be similar (Figure 9 and

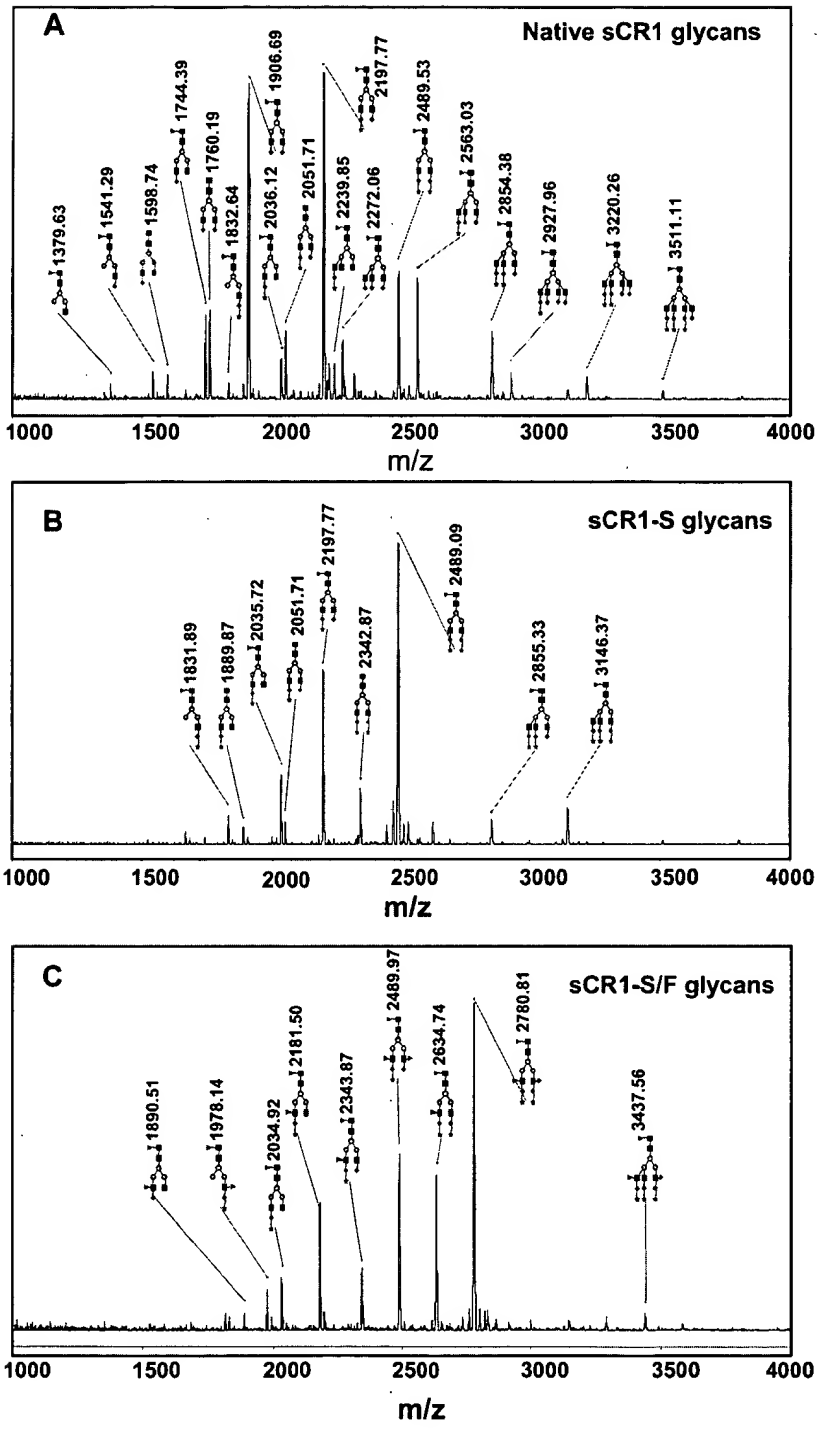


Fig. 7. MALDI-TOF analysis of total glycans from (A) sCR1, (B) sCR1-S, and (C) sCR1-S/F remodeled at the 10-g scale. The blue square is GlcNAc, the yellow filled circle is mannose, the green filled triangle is fucose, the red filled diamond is galactose, and the asterisk is sialic acid.

Table IV), indicating that *in vitro* glycosylation of sCR1 to yield sCR1-S or sCR1-S/F does not significantly impact the complement inhibitory properties of the molecule in the classical pathway.

In vitro *binding to E-selectin*

Figure 10 shows that sCR1-sLe^x and sCR1-S/F bind E-selectin in a concentration-dependent manner. The IC_{50}

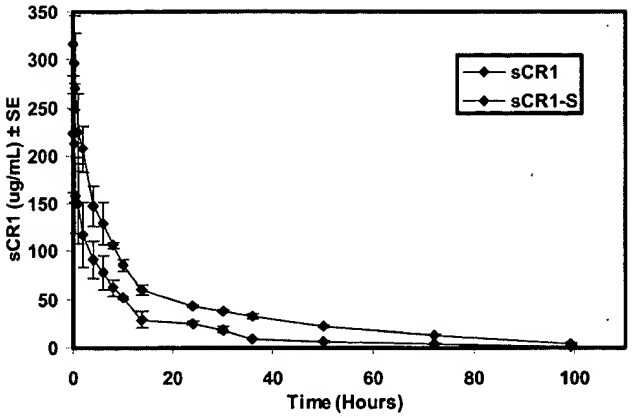


Fig. 8. The concentration of sCR1 and sCR1-S in plasma at various time points following bolus IV injection in rats.

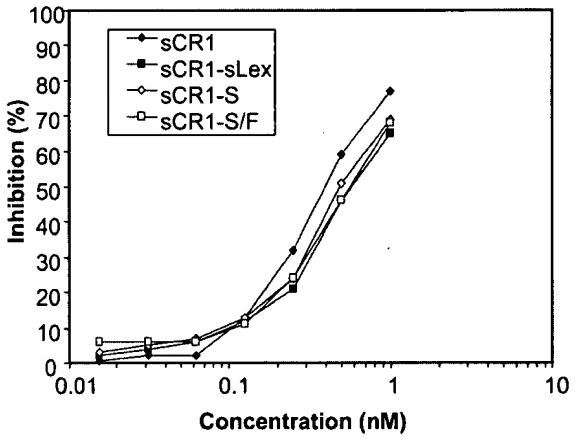


Fig. 9. Inhibition of red cell lysis via the classical pathway as a function of the concentration of sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F.

Table IV. Antihemolytic activity of modified sCR1 and sCR1-sLe^x

	IH_{50} (nM)
sCR1	0.41
sCR1-S	0.48 nM
sCR1-sLe^x	0.59
sCR1-S/F	0.59 nM

for sCR1-sLe^x from this plot is ~5 nM, and for sCR1-S/F ~0.4 nM. The observed 10-fold increase in inhibitory potency presumably is due to enhanced avidity, attributable to the increased density of sLe^x moieties on sCR1-S/F (28/mol) as compared with sCR1-sLe^x (14 per mol) (see Table I). The specificity of this binding was demonstrated by its calcium requirement and by the observation that sCR1

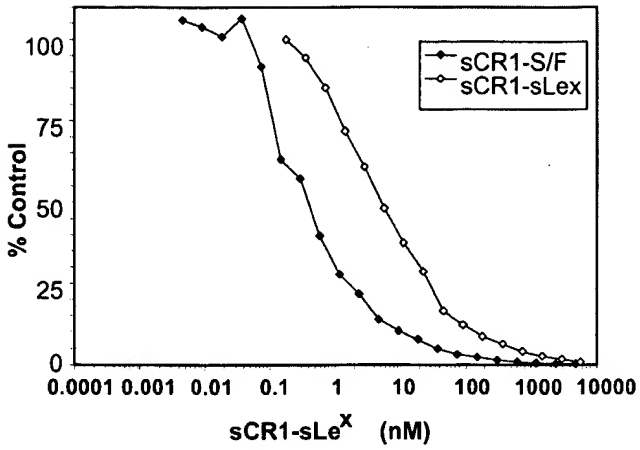


Fig. 10. Inhibition of PAA-sLe^x binding to E-selectin coated microtiter plates in the presence of varying concentrations of sCR1-sLe^x or sCR1-S/F.

(which does not contain any sLe^x structures) does not inhibit E-selectin binding at concentrations as high as 10 μM (data not shown).

Discussion

sCR1, made by standard CHO production methods, possesses predominantly biantennary oligosaccharides that are incompletely sialylated. We previously described an alternately glycosylated form of sCR1 called TP20 or sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), secreted by the FT-VI-expressing LEC11 CHO cell line and bearing sLe^x moieties on a fraction of its N-linked oligosaccharides. In this article we describe *in vitro* enzymatic remodeling of sCR1 by the stepwise application of two soluble recombinant glycosyltransferases in "one pot": The first step adds sialic acid to make sCR1-S, and the second adds fucose to make sCR1-S/F. The product of these glycan remodeling reactions contains an average of 28 sLe^x moieties per mol, as compared with 14 per mol found in CHO cell-produced sCR1-sLe^x.

That the sCR1 protein remains intact under conditions of glycan remodeling was demonstrated by RP-HPLC and SDS–PAGE analyses showing single polypeptides with expected molecular weights for sCR1-S and sCR1-S/F. Evidence for (1) conformational stability under conditions of the *in vitro* glycosylation reactions, and (2) preserved function despite variations in glycan structure, is provided by the observed near equivalence in bioactivity of sCR1, sCR1-S, sCR1-S/F, and CHO-produced sCR1-sLe^x in a standard complement inhibition assay.

The oligosaccharide structures associated with sCR1-S and sCR1-S/F were assessed by a number of methods. FACE profiling demonstrated a more fully sialylated set of glycoforms for sCR1-S as compared with sCR1 and nearly homogeneous, fully sialylated and fucosylated biantennary N-glycans for sCR1-S/F. Sequencing experiments using FACE provided supporting evidence that sialic acid

was linked α2-3 to galactose and that the predominant, single oligosaccharide band derived from sCR1-S/F was $BiNA_2F_2$. The analyses we performed do not establish linkages between the terminal and penultimate sugars that define sLe^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) versus sLe^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-). However, two factors make it likely that the glycans of sCR1-S/F do, in fact, terminate in sLe^x. First, it is known that in CHO cells, N-linked glycans are most commonly formed by β4GalT-1, and hence have the type-2 structure, Galβ1-4GlcNAcβ1- (Lee *et al.*, 2001). Second, the acceptor specificity of FT-VI is known to be restricted to type 2 chains (Costache *et al.*, 1997; Weston *et al.*, 1992).

During optimization of the sialylation reaction, we noted that incubation of sCR1 with either a low concentration of ST3Gal-III (10 mU/ml) for 24 h or a higher concentration (75 mU/ml) for 1 h produced a nearly maximally sialylated product. Even after incubation at the highest concentration of sialyltransferase tested (600 mU/ml for 24 h), a small fraction of monosialylated biantennary species persisted, perhaps due to steric hindrance at particular sites. Improved pharmacokinetics observed for the fully sialylated sCR1-S molecule as compared with sCR1 is probably a consequence of the added sialic acid blocking the interaction of terminal galactosyl residues with hepatic asialoglycoprotein receptors (Stockert, 1995).

We observed that FT-VI at 25 mU/ml fucosylates most sialylated biantennary glycans within 24 h. No significant differences were observed in catalytic activities of FT-VI expressed in the NSO cell line versus *Aspergillus niger* expression systems. The sCR1 polypeptide was shown to be stable following prolonged incubation with enzyme from either source.

In vitro glycosylation of sCR1 at the 10-g scale was carried out at enzyme concentrations selected to ensure nearly complete reaction at each stage. Success with the single experiment reported is consistent with the ability to predict useful scaled-up reaction conditions over a range of at least 40-fold based on mass of starting substrate. Both the ST3Gal-III and FT-VI enzymes used to glycosylate 10 g sCR1 were produced in *A. niger*, an expression system widely used for the manufacture of industrial enzymes in ton quantities. Although further scale-up would require refinement of incubation conditions, it can be estimated from present results that glycosylation of 1 kg of sCR1 might require 40,000 U ST3Gal-III and 20,000 U FT-VI, amounts that seem plausible to produce at reasonable cost in an industrial setting. To our knowledge, this is the largest scale reported enzymatic glycosylation of a glycoprotein to date by several orders of magnitude (Fischer and Dorner, 1998; Nemansky *et al.*, 1995; Paulson *et al.*, 1977; Raju *et al.*, 2001; Thotakura *et al.*, 1994).

The optimized conditions chosen for scale-up were very similar to the conditions used to generate material used for *in vivo* and *in vitro* studies. Compared with sCR1-sLe^x, sCR1-S/F was shown to have twice the number of sLe^x moieties and about a 10-fold higher apparent affinity for binding to E-selectin. This higher affinity presumably results from increased cooperativity in a multivalent binding reaction wherein sLe^x moieties distributed widely over sCR1-S/F engage multiple immobilized E-selectin molecules. In certain clinical situations, the anticomplement inhibitory and anti-inflammatory activity of sCR1-S/F could be effectively targeted via a similar mechanism to sites of inflammation where endothelial cells have been activated and have up-regulated expression of adhesion molecules including P- and E-selectin. sCR1-sLe^x has been shown to be superior to sCR1 in a complement- and selectin-dependent lung injury model (Mulligan *et al.*, 1999), a murine model of ischemic stroke (Huang *et al.*, 1999), moderating skeletal muscle reperfusion injury (Kyriakides *et al.*, 2001a), moderation of acid aspiration injury (Kyriakides *et al.*, 2001b), reducing ischemia/reperfusion injury in rat lung grafts (Schmid *et al.*, 2001), and a myocardial ischemia and reperfusion model in the rat. sCR1-sLe^x significantly reduced myocardial infarct size and was significantly more effective than sCR1 in reducing neutrophil infiltration into the infarction (Zacharowski *et al.*, 1999). It will be interesting to investigate whether sCR1-S/F is even more effective than sCR1-sLe^x in similar animal models.

Materials and methods

Complement proteins, antibodies, enzymes, and other reagents

Purified sCR1 and sCR1-sLe^x were prepared as previously described (Rittershaus *et al.*, 1999). Nucleotide sugars (CMP–sialic acid and GDP-fucose) were manufactured at Neose (Horsham, PA). CMP–sialic acid was prepared from CTP and sialic acid with recombinant CMP NeuAc synthetase (Shames *et al.*, 1991). GDP-fucose was either made from GDP-mannose using GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase/reductase, or purchased from Yamasa (Chiba, Japan). A gene encoding for a truncated, soluble form of ST3Gal-III (rat) was expressed in *A. niger* var. *awamori* dgr246 P2 using a variant of the expression vector pSL 1180 (Ward and Power, 2003). A 30–60% ammonium sulfate pellet was dissolved in 100 mM NaCl, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with 1 M NaCl, 20 mM MES, pH 6. rFT-VI (human) was expressed either in NSO cells or in *A. niger* as described as a soluble protein lacking the transmembrane domain. For the *A. niger* expressed protein, a 30–60% ammonium sulfate pellet was dissolved in 20 mM MES, pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM MES, pH 6. Soluble recombinant E-selectin was purchased from R&D Systems (Minneapolis, MN). Streptavidin–horseradish peroxidase conjugate (SA-HRP) was from Pierce (Rockford, IL), and biotinylated polyacrylamide polymer (PAA-sLe^x) was from GlycoTech (Rockville, MD). Anti-sCR1 monoclonal antibodies 6B1.H12 and 4D6.1 were prepared as previous described (Nickells *et al.*, 1998). Standards and glycosidases used in FACE analyses were from Glyko (Novato, California).

Preparation of sCR1-S

Lyophilized sCR1 (250 mg) was reconstituted and buffer exchanged into 50 mM Tris, 0.15 M NaCl, 0.05% NaN_3,

pH 7.2, using gel filtration columns (PD-10, Amersham Biosciences), and the concentration of sCR1 was adjusted to 5 mg/ml with the same buffer. Following addition of ST3Gal-III (150 mU/ml) and CMP–sialic acid (7 mM) the mixture was incubated at 32°C. A separate aliquot of the reaction mixture to which a trace amount of CMP–[^{14}C]sialic acid was added was incubated in parallel. From this, aliquot samples were withdrawn at various times and fractionated by isocratic HPLC/size-exclusion chromatography at 0.5 ml/min in 45% MeOH, 0.1% trifluoracetic acid (7.8 mm × 30 cm $TSKG2000_{SWXL}$ column, particle size 5 μm, TosoHaas). Incorporation of sialic acid into glycoprotein was calculated from the fraction of counts in the first eluted peak and the known concentration of sugar nucleotide.

Preparation of sCR1-S/F

After the sialylation reaction had proceeded for 48 h, GDP-fucose was added to a final concentration of 7 mM, $MnCl_2$ to 5 mM, and rFT-VI to 0.1 U/ml. A trace amount of GDP-[^{14}C]fucose was added to a separate aliquot, and both reaction mixtures were incubated at 32°C. Chromatography of the radiolabeled mixture as described showed the transfer of ~44 moles/mole sCR1-S after 48 h and 47 moles after 48 h. The product was provisionally designated sCR1-S/F.

Removal of nucleotide sugars and residual glycosyltransferases using ceramic hydroxyapatite and Q Sepharose chromatography

Glycosyltransferases and nucleotide sugars were removed from remodeled sCR1-S and sCR1-S/F by chromatography on ceramic hydroxyapatite (type I; BioRad, Hercules, CA) followed by Q Sepharose (Amersham Biosciences). Purity was assessed by RP-HPLC on a Poros R1/10 column (4.6 mmD/100 mmL, Applied Biosystems, Framingham, MA).

Optimization of sialylation and fucosylation reactions prior to scale-up

sCR1 was thawed slowly at 4°C and buffer exchanged into 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, using a PD10 column. *In vitro* sialylation of sCR1 (5 mg/ml) was evaluated using varying amounts of ST3Gal-III, 5 mM CMP–sialic acid, in the presence of 0.02% sodium azide at 32°C for 24 h. A trace amount of CMP–[^{14}C]sialic acid was added to an aliquot to monitor incorporation of radioactive sialic acid as described.

To the product (sCR1-S) of the reaction performed at a sialyltransferase concentration of 100 mU/ml (still containing the sialylation reagents) was added $MnCl_2$ and GDP-fucose, each to a final concentration of 5 mM, varing amounts of FT-VI, and a trace amount of GDP-[^{3}H]fucose. The resulting reaction mixture was incubated at 32°C for 24 h. Incorporation of radioactive fucose into the product (sCR1-S/F) was monitored as described for sialic acid.

sCR1 remodeling at 10-g scale

Purified sCR1 (10 g) was dialyzed exhaustively at 4°C against 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, adjusted to a concentration of 5 mg/ml with the same buffer, and incubated with ST3Gal-III (200 mU/ml) and CMP-sialic acid (5 mM) for 36 h at 32°C in a final volume of 2 L. After 36 h, an aliquot containing the sialylated product (sCR1-S) was withdrawn for analysis and the following reagents (final concentrations) were added: rFT-VI (100 mU/ml), GDP-fucose (5 mM), $MnCl_2$, (5 mM). After further incubation at 32°C for 36 h, a precipitate (manganese phosphate) was removed by centrifugation at 3000 × *g* for 5 min, and the sialylated and fucosylated product (sCR1-S/F) was stored at −70°C.

Monosaccharide analysis by HPLC

The neutral and amino sugar composition of glycoproteins was determined after trifluoroacetic acid hydrolysis and reductive amination with anthranilic acid by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1994). Sialic acid content was determined after sodium bisulfate hydrolysis and reaction with o-phenylenediamine by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1995).

Carbohydrate analysis by FACE

Carbohydrate sequencing and electrophoresis by FACE (Glyko and ProZyme, San Leandro, CA) was performed as previously described elsewhere (Picard *et al.*, 2000).

Carbohydrate analysis by 2-AA HPLC and MALDI-TOF MS

Glycans were released by PNGaseF and labeled with 2-AA according to the method described by Anumula and Dhume (1998) except that the labeled glycans were purified on cellulose cartridges (Glyko) according to the manufacturer's instructions. 2-AA-labeled N-glycans were analyzed using a Shodex Asahipak NH_2P-50 4D amino column (4.6 mm × 150 mm). The two solvents used for the separation were (A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and (B) 5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water. The column was eluted isocratically with 70% A for 2.5 min, followed by a linear gradient from 70% to 5% A over a period of 97.5 min, and a final isocratic elution with 5% A for 15 min. Eluted peaks were detected using fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 420 nm.

For MALDI-TOF analysis, a small aliquot of the 2-AA-labeled N-glycans was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 μm pore, 47 mm diameter) floating on water. The dialyzed aliquot was dried in a vacuum centrifuge, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water:acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Glycan structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully characterize isobaric structures.

SDS–PAGE

sCR1 samples before and after *in vitro* enzymatic remodeling were separated on 8–16% gradient Tris-glycine

polyacrylamide gels and stained with colloidal blue Coomassie stain. Gels, staining solutions, and molecular weight standards were obtained from Invitrogen (Carlsbad, CA).

Assays of complement regulatory activity

The inhibition of complement-mediated lysis of antibody-sensitized sheep erythrocytes (classical pathway) was assessed as previously described (Scesney *et al.*, 1996).

E-selectin binding assay

E-selectin binding assays were performed according to previously reported methods (Weitz-Schmidt *et al.*, 1996). Flat-bottom 96-well microtiter plates were coated with 5 μg/ml recombinant human E-selectin (R&D Systems) in 150 mM NaCl, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4 (HEPES-buffered saline, HBS). Coated wells were blocked with 2% bovine serum albumin/HBS. Varying concentrations of sCR1 or sCR1-sLe^x were added to the plate. A complex of a biotinylated polyacrylamide polymer containing sLe^x (PAA-sLe^x, GlycoTech) and SA-HRP was prepared. A dilution of this conjugate complex was added to the wells containing sCR1 or sCR1-sLe^x or buffer and incubated for 90 min at room temperature. The wells were washed with HBS/$CaCl_2$ and 3,3′,5,5′-tetramethylbenzidine substrate (KPL) was added to each well. Color was allowed to develop for 15 min, and the reaction was stopped with 2.0 N H_2SO_4. Bound PAA-sLe^x complex was measured by determining the absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Pharmacokinetic analysis in rats

Male Sprague-Dawley rats (~250 g), with in-dwelling jugular vein cannulas were purchased from Taconic (Germantown, NY) or Harlan Sprague Dawley (Indianapolis, IN). The catheters were periodically flushed with 0.9% saline followed by either heparinized glycerol (1:4 glycerol/333 IU heparin/ml) or heparinized saline (333 IU/ml) to ensure patency.

Animals were injected with sCR1 or sCR1-S (10 mg/kg) via the lateral tail vein as a bolus at time 0. Blood samples were obtained at timed intervals from the jugular vein cannula. The levels of sCR1 and sCR1-S present in the plasma samples were measured by a previously described enzyme-linked immunosorbent assay (Rittershaus *et al.*, 1999). Briefly, microtiter plates were coated with anti-sCR1 monoclonal antibody 6B1.H12 and captured sCR1 from a sample was detected with an HRP-conjugated anti-sCR1 monoclonal antibody 4D6.1. Pharmacokinetic data was analyzed using WinNonlin (Pharsight, Mountain View, CA).

Acknowledgments

The authors thank Michelle Richardson and Gang Yan for expert technical assistance.

Abbreviations

2-AA, 2-anthranilic acid; AUC, area under the curve; CHO, Chinese hamster ovary; FACE, fluorophore-assisted carbohydrate electrophoresis; HBS, HEPES-buffered saline; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; RP-HPLC, reversed phase high-pressure liquid chromatography; SA-HRP, streptavidin–horseradish peroxidase; sCR1, soluble recombinant complement receptor type 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References

Anumula, K.R. (1994) Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. *Anal. Biochem.*, **220**, 275–283.

Anumula, K.R. (1995) Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal. Biochem.*, **230**, 24–30.

Anumula, K.R. and Dhume, S.T. (1998) High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology*, **8**, 685–694.

Costache, M., Apoil, P.A., Cailleau, A., Elmgren, A., Larson, G., Henry, S., Blancher, A., Iordachescu, D., Oriol, R., and Mollicone, R. (1997) Evolution of fucosyltransferase genes in vertebrates. *J. Biol. Chem.*, **272**, 29721–29728.

Fischer, B.E. and Dorner, F. (1998) Recombinant coagulation factor IX: glycosylation analysis and *in vitro* conversion into human-like sialylation pattern. *Thromb. Res.*, **89**, 147–150.

Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B., and Rasmussen, J.R. (1991) The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Biotechnology*, **9**, 1347–1355.

Huang, J., Kim, L.J., Mealey, R., Marsh, H.C. Jr., Zhang, Y., Tenner, A.J., Connolly, E.S. Jr., and Pinsky, D.J. (1999) Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. *Science*, **285**, 595–599.

Jenkins, N., Parekh, R.B., and James, D.C. (1996) Getting the glycosylation right: implications for the biotechnology industry. *Nat. Biotechnol.*, **14**, 975–981.

Kallio, E.A., Lemstrom, K.B., Hayry, P.J., Ryan, U.S., and Koskinen, P.K. (2000) Blockade of complement inhibits obliterative bronchiolitis in rat tracheal allografts. *Am. J. Respir. Crit. Care Med.*, **161**, 1332–1339.

Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001a) Moderation of skeletal muscle reperfusion injury by a sLe(x)-glycosylated complement inhibitory protein. *Am. J. Physiol. Cell Physiol.*, **281**, C224–C230.

Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001b) Sialyl Lewis(x) hybridized complement receptor type 1 moderates acid aspiration injury. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **281**, L1494–L1499.

Lasky, L.A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.*, **64**, 113–139.

Lazar, H.L., Bao, Y., Gaudiani, J., Rivers, S., and Marsh, H. (1999) Total complement inhibition: an effective strategy to limit ischemic injury during coronary revascularization on cardiopulmonary bypass. *Circulation*, **100**, 1438–1442.

Lee, J., Sundaram, S., Shaper, N.L., Raju, T.S., and Stanley, P. (2001) Chinese hamster ovary (CHO) cells may express six beta 4-galactosyltransferases (beta 4GalTs). Consequences of the loss of functional beta 4GalT-1, beta 4GalT-6, or both in CHO glycosylation mutants. *J. Biol. Chem.*, **276**, 13924–13934.

Lehmann, T.G., Koeppel, T.A., Kirschfink, M., Gebhard, M.M., Herfarth, C., Otto, G., and Post, S. (1998) Complement inhibition by soluble complement receptor type 1 improves microcirculation after rat liver transplantation. *Transplantation*, **66**, 717–722.

Mulligan, M.S., Warner, R.L., Rittershaus, C.W., Thomas, L.J., Ryan, U.S., Foreman, K.E., Crouch, L.D., Till, G.O., and Ward, P.A. (1999)

Endothelial targeting and enhanced antiinflammatory effects of complement inhibitors possessing sialyl Lewis x moieties. *J. Immunol.*, **162**, 4952–4959.

Naka, Y., Marsh, H.C., Scesney, S.M., Oz, M.C., and Pinsky, D.J. (1997) Complement activation as a cause for primary graft failure in an isogeneic rat model of hypothermic lung preservation and transplantation. *Transplantation*, **64**, 1248–1255.

Nemansky, M., De Leeuw, R., Wijnands, R.A., and Van Der Eijnden, D.H. (1995) Enzymic remodelling of the N- and O-linked carbohydrate chains of human chorionic gonadotropin. *Eur. J. Biochem.*, **227**, 880–888.

Nickells, M., Hauhart, R., Krych, M., Subramanian, V.B., Geoghegan-Barek, K., Marsh, H.C. Jr., and Atkinson, J.P. (1998) Mapping epitopes for 20 monoclonal antibodies to CR1. *Clin. Exp. Immunol.*, **112**, 27–33.

Paulson, J.C., Hill, R.L., Tanabe, T., and Ashwell, G. (1977) Reactivation of asialo-rabbit liver binding protein by resialylation with β-D-galactoside α2-6 sialyltransferase. *J. Biol. Chem.*, **252**, 8624–8628.

Picard, M.D., Pettey, C.L., Marsh, H.C., and Thomas, L.J. (2000) Characterization of N-linked oligosaccharides bearing sialyl Lewis x moieties on an alternatively glycosylated form of soluble complement receptor type 1 (sCR1). *Biotechnol. Appl. Biochem.*, **31**(1), 5–13.

Pratt, J.R., Hibbs, M.J., Laver, A.J., Smith, R.A., and Sacks, S.H. (1996) Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat. *Am. J. Pathol.*, **149**, 2055–2066.

Pruitt, S.K., Bollinger, R.R., Collins, B.H., Marsh, H.C., Levin, J.L., Rudolph, A.R., Baldwin, W.M., and Sanfilippo, F. (1997) Effect of continuous complement inhibition using soluble complement receptor type 1 on survival of pig-to-primate cardiac xenografts. *Transplantation*, **63**, 900–902.

Raju, T.S., Briggs, J.B., Chamow, S.M., Winkler, M.E., and Jones, A.J.S. (2001) Glycoengineering of therapeutic glycoproteins: *in vitro* galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. *Biochemistry*, **40**, 8868–8876.

Rittershaus, C.W., Thomas, L.J., Miller, D.P., Picard, M.D., Geoghegan-Barek, K.M., Scesney, S.M., Henry, L.D., Sen, A.C., Bertino, A.M., Hannig, G., and others. (1999) Recombinant glycoproteins that inhibit complement activation and also bind the selectin adhesion molecules. *J. Biol. Chem.*, **274**, 11237–11244.

Scesney, S.M., Makrides, S.C., Gosselin, M.L., Ford, P.L., Andrews, B.M., Hayman, E.G., and Marsh, H.C. (1996) A soluble deletion mutant of the human complement receptor type 1, which lacks the C4b binding site, is a selective inhibitor of the alternative complement pathway. *Eur. J. Immunol.*, **26**, 1729–1735.

Schmid, R.A., Hillinger, S., Hamacher, J., and Stammberger, U. (2001) TP20 is superior to TP10 in reducing ischemia/reperfusion injury in rat lung grafts. *Transplant. Proc.*, **33**, 948–949.

Shames, S.L., Simon, E.S., Christopher, C.W., Schmid, W., Whitesides, G.M. and Yang, L.L. (1991) CMP-N-acetylneuraminic acid synthetase of *Escherichia coli*: high level expression, purification and use in the enzymatic synthesis of CMP-N-acetylneuraminic acid and CMP-neuraminic acid derivatives. *Glycobiology*, **1**, 187–191.

Stammberger, U., Hamacher, J., Hillinger, S., and Schmid, R.A. (2000) sCR1sLe^x ameliorates ischemia/reperfusion injury in experimental lung transplantation. *J. Thorac. Cardiovasc. Surg.*, **120**, 1078–1084.

Stockert, R.J. (1995) The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol. Rev.*, **75**, 591–609.

Thotakura, N.R., Szkudlinski, M.W., and Weintraub, B.D. (1994) Structure-function studies of oligosaccharides of recombinant human thyroptrophin by sequential deglycosylation and resialylation. *Glycobiology*, **4**, 525–533.

Ward, M. and Power, S.D. (2003) DNA sequences, vectors, and fusion polypeptides for secretion of polypeptides in filamentous fungi. US Patent no. 6,590,078 B2.

Weisman, H.F., Bartow, T., Leppo, M.K., Marsh, H.C., Carson, G.R., Concino, M.F., Boyle, M.P., Roux, K.H., Weisfeldt, M.L., and Fearon, D.T. (1990) Soluble human complement receptor type 1: *in vivo* inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science*, **249**, 146–151.

Weitz-Schmidt, G., Stokmaier, D., Scheel, G., Nifant'ev, N.E., Tuzikov, A.B., and Bovin, N.V. (1996) An E-selectin binding assay based on a polyacrylamide-type glycoconjugate. *Anal. Biochem.*, **238**, 184–190.

Weston, B.W., Smith, P.L., Kelly, R.J., and Lowe, J.B. (1992) Molecular cloning of a fourth member of a human α(1,3)fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis x, sialyl Lewis x, and difucosyl sialyl Lewis x epitopes. *J. Biol. Chem.*, **267**, 24575–24584.

Williams, J.P., Pechet, T.T., Weiser, M.R., Reid, R., Kobzik, L., Moore, F.D. Jr., Carroll, M.C., and Hechtman, H.B. (1999) Intestinal reperfusion injury is mediated by IgM and complement. *J. Appl. Physiol.*, **86**, 938–942.

Zacharowski, K., Otto, M., Hafner, G., Marsh, H.C. Jr., and Thiemermann, C. (1999) Reduction of myocardial infarct size with sCR1sLe(x), an alternatively glycosylated form of human soluble complement receptor type 1 (sCR1), possessing sialyl Lewis x. *Br. J. Pharmacol.*, **128**, 945–952.

Zhang, A., Potvin, B., Zaiman, A., Chen, W., Kumar, R., Phillips, L., and Stanley, P. (1999) The gain-of-function Chinese hamster ovary mutant LEC11B expresses one of two Chinese hamster FUT6 genes due to the loss of a negative regulatory factor. *J. Biol. Chem.*, **274**, 10439–10450.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 275, No. 32, Issue of August 11, pp. 24237–24245, 2000
Printed in U.S.A.

Human α1,3/4 Fucosyltransferases

CHARACTERIZATION OF HIGHLY CONSERVED CYSTEINE RESIDUES AND *N*-LINKED GLYCOSYLATION SITES*

Received for publication, February 1, 2000, and in revised form, May 10, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M000888200

Eric H. Holmes‡§, Ten-Yang Yen§¶, Scott Thomas§¶, Rajesh Joshi¶, Anton Nguyen¶, Tracy Long¶, François Gallet‖, Abderrahman Maftah, Raymond Julien‖, and Bruce A. Macher¶ ‡‡**

*From the ‡Department of Cell Surface Biochemistry, Molecular Medicine, Northwest Hospital, Seattle, Washington 98134, the ¶Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, California 94132, and ‖Génétique Moléculaire Animale, UMR 1061, INRA, and **Glycobiologie et Biotechnologie, UPRES 1074, Faculté des Sciences, Université de Limoges, 123 Avenue Albert Thomas, 87060 Limoges, France*

Human α1,3 fucosyltransferases (FucTs) contain four highly conserved cysteine (Cys) residues, in addition to a free Cys residue that lies near the binding site for GDP-fucose (Holmes, E. H., Xu, Z., Sherwood, A. L., and Macher, B. A. (1995) *J. Biol. Chem.* 270, 8145–8151). The participation of the highly conserved Cys residues in disulfide bonds and their functional significance were characterized by mass spectrometry (MS) analyses and site-directed mutagenesis, respectively. Among the human FucTs is a subset of enzymes (FucT III, V, and VI) having highly homologous sequences, especially in the catalytic domain, and Cys residues in FucT III and V were characterized. The amino acid sequence of FucT III was characterized. Peptides containing the four conserved Cys residues were detected after reduction and alkylation, and found to be involved in disulfide bonds. The disulfide bond pattern was characterized by multiple stage MS analysis and the use of Glu-C protease and MS/MS analysis. Disulfide bonds in FucT III occur between Cys residues (Cys^{81} to Cys^{338} and Cys^{91} to Cys^{341}) at the N and C termini of the catalytic domain, bringing these ends close together in space. Mutagenesis of highly conserved Cys residues to Ser in FucT V resulted in proteins lacking enzymatic activity. Three of the four mutants have molecular weights similar to wild type enzyme and maintained an ability to bind GDP, whereas the other (Cys^{104}) produced a series of lower molecular weight bands when characterized by Western blot analysis, and did not bind GDP. FucTs have highly conserved, potential *N*-linked sites, and our mass spectrometry analyses demonstrated that both *N*-linked sites are modified with oligosaccharides.

α1,3/4 fucosyltransferases (FucTs)[1] have been cloned from a variety of species and share significant sequence homology (see Refs. 1–5 and references therein). Among the conserved residues in FucTs (including human, mouse, chicken, and zebra fish) are four cysteine (Cys) residues (Fig. 1). Two of these Cys residues are located near the N terminus and two near the C terminus of the catalytic domain. The Cys residues found at the C terminus also are conserved in FucTs from *Caenorhabditis elegans* (6).

Among the human FucTs, FucTs III, V, and VI share substantial sequence homology. Within the catalytic domain only about 20 out of 300 amino acids vary among the three proteins. In addition, domain swapping experiments by our group (7) and Lowe and co-workers (9) have demonstrated that chimeric proteins composed of partial sequence from each of these FucTs are active, indicating that the minor differences in their amino acid sequences does not result in major alterations in their overall structure. Therefore, we have used two (FucT III and V) of these highly homologous proteins in the present study to evaluate the structure and functional significance of the highly conserved Cys residues.

Protein chemistry experiments, coupled with mass spectrometry analyses, have been used to locate all peptides containing Cys residues in human FucT III, allowing them to be assigned either as being involved in a disulfide bond or as a free Cys residue, and identifying which Cys residues are bound to each other in disulfide linkages. The results support our (7) previously stated hypothesis that amino acids affecting acceptor substrate specificity of human FucT III and V, located near the N and C termini of the catalytic domain, are brought close together in space by disulfide bonds between these highly conserved Cys residues.

To investigate the importance of these Cys residues, we have mutated each of these Cys residues in human FucT V and evaluated the activity and other properties of each resulting protein construct. The results demonstrate that these residues affect enzyme activity, but not the interaction of the protein with GDP-Fuc in three of the four cases. In the case of one of the mutant constructs (Cys^{104}) protein folding/stability is altered compared with the wild type protein.

The amino acid sequences of FucTs have been predicted on the basis of cDNA sequences, but none of the amino acid se-

* This work was supported by Research Grant CA70740 from the NCI, National Institutes of Health (to E. H. H.), by National Institutes of Health Grant RR11805 (Research Infrastructure in Minority Institution) and National Science Foundation grant MCB-9816780 (both to B. A. M.), and by the French GTrec Network. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: Office of Research and Sponsored Programs, San Francisco State University, 1600 Holloway Ave., San Francisco, CA 94132. Tel.: 415-338-7091; Fax: 415-338-2493; E-mail: macher@sfsu.edu.

[1] The abbreviations used are: FucT, fucosyltransferase; ESI, electrospray ionization; H-type II, Fucα1,2Galβ1,4GlcNAc; LC, liquid chromatography; MS, mass spectrometry; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; type I, Galβ1,3GlcNAc; MS^3, multiple stage mass spectrometry; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PNGase F, peptide:*N*-glycanase F; MOPS, 4-morpholinepropanesulfonic acid; ASA, azidosalicyclic acid; PEO, polyethylene oxide.

quences have been confirmed directly. Furthermore, little is known about sites of posttranslational modifications. Among the potential sites for posttranslational modification in FucTs, are Asn residues that may be substituted with *N*-linked glycans (Ref. 8 and references therein). Among FucTs III, V, and VI, there are two highly conserved *N*-linked sites, plus others that are less highly conserved (*i.e.* they occur in FucTs V and VI, but not FucT III). From previous analyses (7, 9), it is clear that some, but not all, of these sites are posttranslationally modified. In the current study, a combination of proteolytic digestions and MS/MS analyses have been used to analyze the amino acid sequence of FucT III. This methodology in combination with PNGase F treatment has been used to locate Asn residues in FucT III that are glycosylated.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled GDP-Fuc was purchased from Calbiochem (San Diego, CA), and GDP-[^{3}H]Fuc was purchased from NEN Life Science Products. The following items were obtained from HyClone Laboratories Inc. (Logan, UT): Dulbecco's modified Eagle's medium, phosphate-buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), penicillin, streptomycin, and fetal calf serum. Rabbit IgG-agarose, anti-goat IgG-alkaline phosphatase conjugate, goat IgG, bovine serum albumin, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, and formamide were obtained from Sigma. PEO-maleimide-activated biotin was purchased from Pierce. PNGase F and chymotrypsin were purchased from Roche Molecular Biochemicals, and trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). SP-Sepharose was from Amersham Pharmacia Biotech, and the GDP affinity resin was a gift from Dr. Ole Hindsgaul (Department of Chemistry, University of Alberta, Alberta, Canada). All other chemicals were obtained from commercial sources and were of the highest purity available.

FucT V Mutants—The truncated, wild type FucT V was described previously (7) and used as a template to generate the Cys → Ser mutants at Cys^{94}, Cys^{104}, Cys^{351}, and Cys^{354} and the double mutant Cys^{351} → Ser/Cys^{354} → Ser by PCR. To facilitate cloning of the mutants, a modified form of the FucT V coding sequence containing a *Not*I site at nucleotide base 984 of the FucT V sequence was generated by PCR mutagenesis. This new restriction site was used in PCR mutagenesis (7, 10) experiments to generate mutations, which produced the Cys^{351} → Ser, Cys^{354} → Ser, and Cys^{351} → Ser/Cys^{354} → Ser FucTs. Mutations at the 5′ end of the FucT V coding region (Cys^{94} and Cys^{104} of FucT V) were introduced by recombinant PCR as described previously (7), and the PCR products were subcloned between the 5′ terminus (*Eco*RI site), and the *Eco*RV site (nucleotide base 373–378 in FucT V) that exists in the FucT V coding region.

Recombinant PCR was used to create Cys^{94} → Ser and Cys^{104} → Ser mutants. The flanking primers (A and D) were used in the second round of PCR to create the full-length PCR product that was inserted into pPROTA. The underlined segments are restriction sites that were used in subcloning or to screen for recombinants (GAATTC (*Eco*RI), ATTAAT (*Ase*I), GGTACC (*Kpn*I), GATATC (*Eco*RV)). Standard PCR was used to create Cys^{351} → Ser, Cys^{354} → Ser, and Cys^{351} → Ser/Cys^{354} → Ser mutants. The underlined segments are restriction sites that were used in subcloning or to screen for recombinants. TGCGGCCGC (*Not*I), GTCGAC (*Sal*I), AAGCTT (*Hin*dIII). Each mutant coding region was verified by DNA sequencing.

The primers used are: primer A forward, 5′-GGAATTCGCTATTAATCCTGCTGTGGACGTGGCCTTTT-3′; primer B reverse, 5′-CGCGGCGCCGGGTACCATCTCTGAGGAGCGGGGCAGAGCCAC-3′; primer C forward, 5′-GTGGCTCTGCCCCGCTCCTCAGAGATGGTACCCGGCGCCGCG-3′; primer D reverse, 5′-CATGATATCCCAGTGGTGCACGAT-3′; primer E reverse, 5′-GTCGGCAGTGATGTTGGAGTCCGCGGCGCCGGGTACCATCTC-3′; primer F forward, 5′-GAGATGGTACCCGGCGCCGCGGACTCCAACATCACTGCCGAC-3′; primer G forward, 5′-CGCTGCGGCCGCGCTCCTTCAGCTGGGCACTGGCTTTCTCCAAAGCTTGCTGG-3′; primer H reverse, 5′-GCCGACGTCGACTCAGGTGAACCAAGCCGCTATGCTGCGCACCGT-3′; primer I forward, 5′-CGCTGCGGCCGCGCTCCTTCAGCTGGGCACTGGCTTTCTGCAAAGCTTCCTGGAAG-3′; primer J forward, 5′-CGCTGCGGCCGCGCTCCTTCAGCTGGGCACTGGCTTTCTCCAAAGCTTCCTGGAAG-3′.

The resulting plasmids were propagated in the JM109 strain of *E. coli* and transfected into COS-7 cells. Wild type and mutant proteins were expressed as soluble proteins with an N-terminal protein A, IgG binding domain (11). The fusion proteins were purified from the cell culture media by IgG-agarose column chromatography. Recombinant FucTs were detected and quantified via Western blot analysis (16).

Fucosyltransferase Assays—The standard reaction mixture contained: 50 mM MOPS/NaOH buffer, pH 6.5, 6.25 mM $MnCl_2$, 0.05% bovine serum albumin, 3.0 nmol of GDP-Fuc, 0.01 μCi of GDP-[^{3}H]Fuc, 20 nmol of acceptor, and 5 μl of the enzyme, and reactions were terminated by adding 400 μl of water. The reaction product was separated from substrate by reverse phase chromatography (Sep-Pak C_{18}) and quantified as described previously (12).

Preparation of ^{125}I-GDP-hexanolamine-ASA—GDP-hexanolamine-ASA was iodinated (see Ref. 13) in reaction mixtures that contained two IODOBEADS (Pierce), 5 μmol sodium phosphate buffer, pH 7.0, 1 mCi of $Na^{125}I$, and H_2O in a total volume of 60 μl. At the end of 15 min, 0.6 μmol of GDP-hexanolamine-ASA in 35 μl of H_2O was added and incubated for an additional 15 min at room temperature. The reaction was stopped by removal of the solution from the IODOBEADS and labeled GDP-hexanolamine-ASA used in photolabeling experiments.

Photoaffinity Labeling of Beaded Enzyme—Wild type FucT V and FucT V Cys mutant constructs expressed in COS-7 cells and adsorbed onto IgG-agarose beads were suspended in a 25% bead slurry in PBS. Aliquots (10 μl), each containing 350–540 ng of expressed protein, were dispensed in reaction mixtures, which also contained 0.5 μmol of sodium phosphate buffer, pH 7.0, 0.625 μmol of ^{125}I-GDP-hexanolamine-ASA (approximately 1×10^7 cpm/nmol), with or without 20 μmol of GDP-Fuc, and H_2O in a total volume of 50 μl in wells of a round-bottomed 96-well plate. The reaction mixtures were allowed to stand for 30 min at room temperature in the dark prior to photolysis at 254 nm for 1 min with a hand-held UV lamp placed directly above the wells. The beads were quantitatively transferred to 0.5-ml Eppendorf tubes, washed in PBS, and SDS gel sample buffer (Bio-Rad; 30 μl) was added. The tubes were heated at 100 °C for 10 min and centrifuged, and 25-μl aliquots were electrophoresed on 8.5% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Blue, destained, and dried. The dried gels were exposed to X-Omat AR film to locate labeled protein. Labeled protein bands were cut out and quantified by counting in a γ counter.

FucT III Expression and Purification—FucT III was expressed in *Pichia pastoris* as described previously (14). To purify FucT III, pellets were resuspended to an OD_{600} = 100 with 50 mM cacodylate, pH 7.0, 0.1 mM dithiothreitol. Lysis was achieved by glass bead beating using a bead beater. The beating chamber was filled with a 50/50 mixture of glass beads (Sigma acid-washed; 425–600 μm) and the resuspended pellet. Four lysis cycles were used to free the enzyme from the pelleted material (1 min beating and 2 min icing). Cell debris was removed by centrifugation at 27,500 × *g* for 1 h, and the supernatant was used as the enzyme source. The enzyme was purified in a two-step procedure that included cation exchange (SP-Sepharose) and a GDP-resin affinity chromatographic procedures. The crude pellet lysate was loaded onto the SP-Sepharose column, which was equilibrated in 50 mM cacodylate, pH 7.0 buffer containing 1.0 mM dithiothreitol. The column (approximately 50 ml) was washed with 200 ml of the loading buffer, and the enzyme was eluted with loading buffer containing 0.3 M NaCl. Fractions containing FucT III activity were pooled and loaded onto a 4-ml GDP-hexanolamine affinity resin (~3 μmol of ligand/ml of resin), which was equilibrated with a loading buffer containing 10 mM cacodylate, pH 7.0, 10 mM MgCl. The sample was applied at a flow rate of 0.2 ml/min and the column washed with 12 ml of loading buffer at the same flow rate. FucT III active fractions were eluted from the column with loading buffer containing 2 mM GDP at 0.2 ml/min. Fractions were analyzed for enzyme activity, and proteins by SDS-PAGE.

Modification of Cys Residues—One-ml fractions of FucT III (~50 ng/μl) from the GDP-affinity column were concentrated by Centricon filtration to 40 μl (1 μg/μl, 10 mM cacodylate, 10 mM $MgCl_2$, 2 mM GDP at pH 7.0). A 5-μl sample of the concentrated FucT III (~ 5 μg) was incubated with a 20-fold molar excess of PEO-maleimide-activated biotin for 30 min in the dark at room temperature (total volume 6 μl). Biotin-labeled FucT III was denatured with urea (8.4 M) in a final volume of 9.1 μl for 30 min before tryptic digestion.

Digestion with Trypsin—The concentration of urea in the denatured FucT III sample was reduced to 2 M by adding water. Trypsin (1/5 ratio w/w of trypsin/protein) was added and the mixture (36.6 μl) was incubated overnight at 37 °C.

Endoproteinase Glu-C Digestion—Fractions of the tryptic digest of biotin modified FucT III eluted at 34–38 min were collected and dried by vacuum, and dissolved in 20 μl of ammonium bicarbonate buffer (100 mM, pH 7.9), and the endoproteinase Glu-C (1/10, w/w, enzyme/protein) was added. Digestion was carried out overnight at 37 °C.

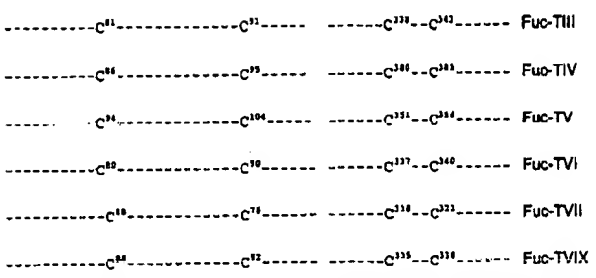


FIG. 1. **Alignment of conserved Cys residues (labeled C) in human α1,3/4 fucosyltransferases.**

PNGase F Digestion—PNGase F was dissolved in 100 mM sodium phosphate, 25 mM EDTA at pH 7.2 at a concentration of 200 units/ml. PNGase F digestion was done on tryptic digests of FucT III by adding PNGase F to a final concentration of 20 units/ml, and the mixture was incubated overnight at 37 °C.

Sequence Analysis of Peptides by HPLC/MS/MS—LC/MS/MS analysis was performed using an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA) with a modified electrospray ionization (ESI) source. A positive voltage of 3 kV was applied to the electrospray needle, and the temperature of the stainless steel heating capillary was maintained at 220 °C. A N_2 sheath flow (65 scale) was applied to stabilize the ESI signal. The voltage at the exit of the heating capillary and the tube lens was held at 13 and 5 V, respectively, to minimize the source-induced dissociation and optimize the ESI signal of the analyte. The ion injection was controlled by automatic gain control to avoid the space charge effects. The full scan mass spectrum was acquired from m/z = 300 to m/z = 2000. The MS/MS experiments were executed with a relative collision energy of 38%. The LC/MS analysis was conducted using a 1050 HPLC system (Hewlett-Packard, Palo Alto, CA) coupled to the LCQ. The HPLC system was operated at a flow rate of 0.25 ml/min. The mobile phase was split before the injector by a tee-connector. One end of the tee was connected to a capillary C18 column (150 × 0.18 mm; Nucleosil, 5-μm particle size) and a flow rate of 2 μl/min was established. The enzymatically digested peptides were eluted from the column using 0.5% formic acid in water (mobile phase A) and 0.5% formic acid in acetonitrile (mobile phase B) with a three-step linear gradient of 5–10% B in the first 10 min, 10–35% B in the next 40 min and 35–40% B in the last 5 min. The LC/MS/MS analysis was accomplished using an automated data acquisition procedure, in which a cyclic series of three different scan modes were performed. Data acquisition was conducted using the full scan mode to obtain the most intense peak (signal > 1.5 × 10^5 counts) as the precursor ion, followed by a high resolution zoom scan mode to determine the charge state of the precursor ion and an MS/MS scan mode to determine the structural fragment ions of the precursor ion. The resulting MS/MS spectra were then searched against a protein data base (Owl) by Sequest to confirm the sequence of tryptic peptides. Multiple stage (MS^3) analysis was performed by selecting a fragment ion from the MS/MS analysis as the precursor ion to generate the MS^3 spectrum. The relative collisional energy for the MS/MS and MS^3 analyses was set from 30% to 43%.

RESULTS

Six human FucTs have been cloned (FucT III to FucT VII and FucT IX). A comparison of the distribution of Cys residues among these enzymes demonstrates that there are four highly conserved Cys residues (see Fig. 1), which are also highly conserved in FucTs from other species. The importance of these conserved Cys residues for protein structure (*e.g.* disulfide bond formation) and enzyme activity is unknown. Therefore, a series of protein chemistry/mass spectrometry and site-directed mutagenesis studies have been carried out to provide the first information on the structure/function relationships of these highly conserved Cys residues.

Amino Acid Sequence Analysis of FucT III—The availability of a yeast expression system for FucT III provided an opportunity to obtain a sufficient quantity of enzyme for a complete characterization of the protein's amino acid sequence, its disulfide bond pattern and the location of *N*-linked glycosylation. FucT III was expressed as a soluble protein in *P. pastoris* (14) and purified in a two-step process that yielded a single Coomassie-stained band by SDS-PAGE. Fractions containing this protein band were highly active (specific activity: 1200 nmol/min mg protein) for fucosyltransferase activity with a type I acceptor. In-gel tryptic digestion products of the band detected on the SDS-PAGE contained peptides derived from the FucT III sequence, and there was no conclusive evidence for the presence of any other protein contaminants (data not shown). A complete peptide sequence analysis of the purified protein solution (reduced with dithiothreitol and alkylated with iodoacetamide) was done using a combination of tryptic digestion and tandem mass spectrometry analysis. More than 95% of the amino acid sequence predicted from the cDNA for FucT III was confirmed by using the protein data base searching program, Sequest (data not shown). Two peptides (amino acids 152–160 and 161–189) that contain an *N*-linked consensus sequence were not detected. However, after treatment with PNGase F, modified (*i.e.* peptides with Asn converted to Asp) peptides corresponding to these sequences were detected. MS/MS analysis confirmed that the detected amino acid sequence contained Asp in place of Asn. Thus, the peptide containing amino acids 152–160 (YF(N → D)LTMSYR) gave a y_7 ion with m/z = 885.4 instead of 884.4 predicted for the peptide with Asn present, and the peptide containing amino acids 161–189 (SDSDIFTPYGWLEPWSGQPAHPPL(N → D)LSAK) gave a y_8 ion with m/z = 840.2 instead of 839.2 predicted for the peptide with Asn present. Thus, both of the highly conserved (found in FucT III, V, and VI) *N*-linked glycosylation sites are glycosylated when FucT III is expressed in the yeast system.

Identification of Free and Disulfide-bonded Cys Residues—Purified FucT III was reacted with PEO-maleimide-activated biotin to label any free Cys residues (one was predicted to occur at Cys^{143}; see Ref. 15), denatured and digested with trypsin under non-reducing conditions. The tryptic digest was separated by liquid chromatography and analyzed for peptides containing modified Cys residues by ESI-MS/MS. A triply charged ion (Fig. 2*A*) for the Cys-containing peptide (132–151) at m/z = 1002.3 and a doubly charged ion at m/z 1503.0 were detected. The MS/MS analysis (Fig. 2*B*) of the ion specie at m/z 1002.3 conclusively demonstrated that Cys^{143} was conjugated to the biotin reagent. This is illustrated by the observed mass of the fragment ions (y_{12} and y_{13}, doubly charged ion at m/z 959.6 and 1008.1, respectively) containing the biotin modified Cys residue. These ions have a mass that is 525.2 Da greater than expected if the Cys residue were unmodified; this mass corresponds to that expected for the peptide containing a modified Cys residue. Thus, the results presented in the MS/MS spectrum clearly show that Cys^{143} is a free Cys residue in the native protein as we previously predicted. This Cys residue is known to lie in or near the GDP-Fuc binding site (15). No other biotinylated Cys-containing peptides were detected, indicating that the four other highly conserved Cys residues are involved in disulfide bridges.

To directly demonstrate that these Cys residues are indeed involved in disulfide bonds and determine how these Cys residues are linked, the m/z values of possible disulfide-linked peptide combinations were calculated and the data generated from the LC/ESI-MS/MS analysis of a nonreduced preparation of FucT III was searched for possible matches. The search identified ions (detected as both triply m/z 1171.9 and quadruply m/z 879.1 charged species) that would result if the three Cys-containing peptides (amino acids 81–97, 330–339, and 340–343; see Fig. 3*A*) were eluted as a disulfide-linked, tripeptide complex. MS/MS analysis of the quadruply charged ion at m/z 879.1 (data not shown) further supported the presence of a complex of the three Cys-containing peptides with the most

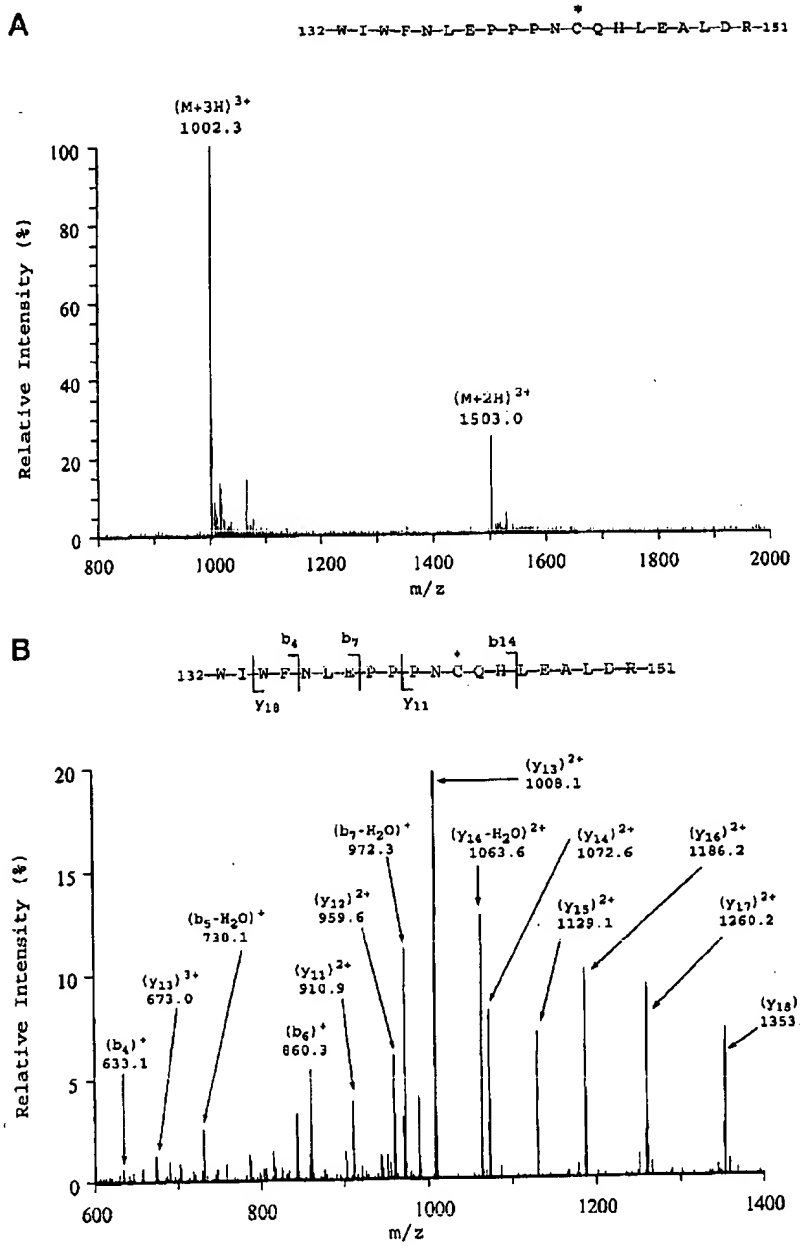


FIG. 2. *A*, the full scan mass spectrum of the modified Cys^{143}-containing tryptic peptide (amino acids 132–151) of FucT III at m/z 1503.0 for the doubly charged ion and m/z 1002.3 for the triply charged ion. *B*, the corresponding MS/MS spectrum for m/z 1002.3 contains both b- and y-dominant (b_n, n = 4–7 and 14; y_n, n = 11–18) fragment ions, confirming the sequence and Cys modification. The position of the modified Cys residue is designated with an *asterisk* (*).

abundant fragments generated by a loss of amino acids from N terminus of peptide 330–339. Low energy collision-induced dissociation resulted in a small amount of S-S bond dissociation, which was only observed at m/z 1288.3 (y_{12}+S).

Interestingly, the MS/MS spectrum of the triply charged ion for the tripeptide complex at m/z 1172.0 (see Fig. 4) shows that the dominant fragment ions are generated from a preferential cleavage at the amide bond of Pro^{86} within the peptide containing Cys^{81} and Cys^{91}. This MS/MS fragmentation pattern is consistent with previous studies of Pro-containing peptides by Loo *et al.* (16), who observed a similar pattern with peptides derived from several proteins. The fragment ions, m/z 1750.3 (b_5Y_{10}) and 881.1 ($y_{12}Y_4$*), correspond to singly and doubly charged ions of disulfide-containing peptides, respectively. The ion at m/z 881.1 is predicted to result from the combination of the two peptides containing Cys^{91} and Cys^{343} (peptides containing amino acids 86–97 and 340–343, respectively), and the ion at m/z 1759.1 is predicted to result from the combination of the two peptides containing Cys^{81} and Cys^{338} (peptides containing amino acids 81–85 and 330–339, respectively). These results suggest that the disulfide linkage patterns are Cys^{81}-Cys^{338} and Cys^{91}-Cys^{343}. Two additional sets of experiments were used to verify the identity of these ion species as the tripeptide complex and to validate the assigned disulfide bond pattern.

Analysis of the Tripeptide Complex by MS^3—An ion trap mass spectrometer is capable of performing multiple stage MS/MS analysis to obtain structural information. MS^3 analysis for the MS/MS fragment ions, m/z 881.1 and 1750.3 was employed to confirm the assignment of the Cys^{91}-Cys^{343} and Cys^{81}-Cys^{338} disulfide bonding pattern. The dominant product ion in the MS^3 spectrum of m/z 881.1 ($y_{12}Y_4$*) is the doubly

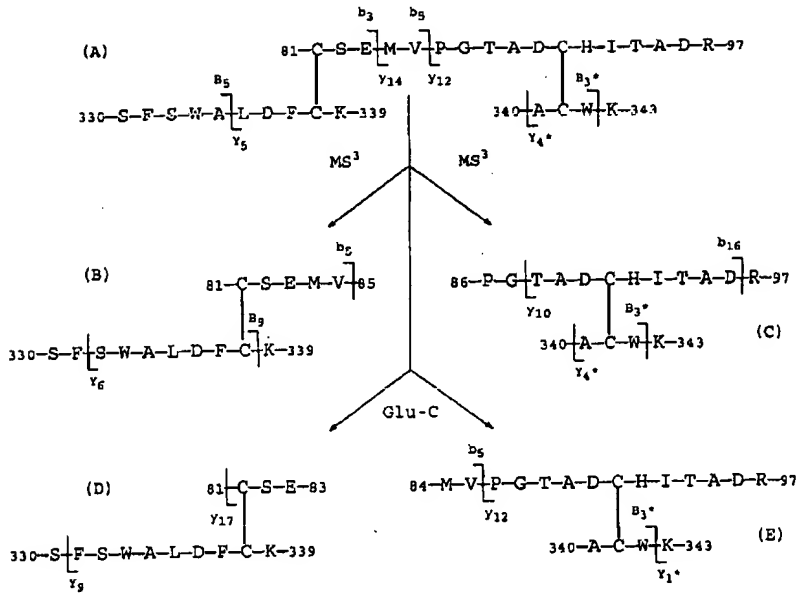


FIG. 3. **Scheme of (*A*) the disulfide-linked tripeptide from FucT III and the products observed in the MS³ (*B* and *C*) and Glu-C experiments (*D* and *E*).**

	Fragment	Charge	Mass
1	B_5-H_2O	+	561.0
2	Y_7Y_4*	2+	660.4
3	$b_{12}Y_4$*	2+	872.0
4	$Y_{12}Y_4$*	2+	881.1
5	b_5Y_3	+	944.1

	Fragment	Charge	Mass
6	M+3H-H_2O	3+	1165.4
7	$Y_3Y_{17}B_3$*	2+	1280.0
8	$Y_3Y_{17}Y_4$*	2+	1353.4
9	$Y_4Y_{17}Y_4$*	2+	1410.3
10	$Y_5Y_{17}Y_4$*	2+	1467.0
11	$Y_{10}Y_{17}$+S	2+	1520.5
12	$b_{16}Y_{10}Y_4$*	2+	1669.8
13	b_5Y_{10}	+	1750.3
14	$Y_{12}Y_4$*	+	1761.5

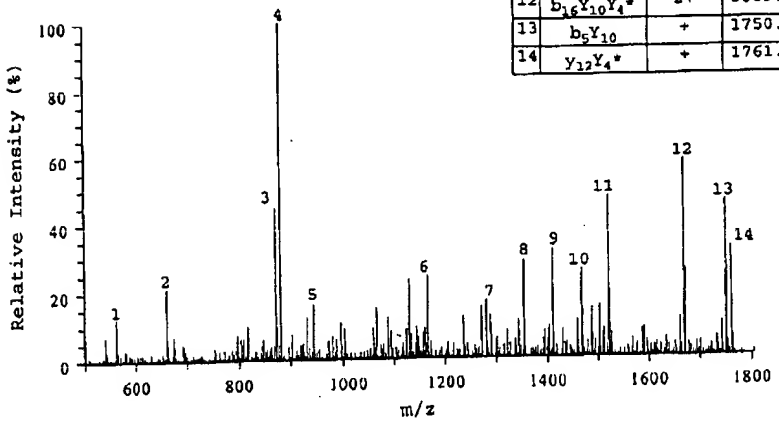


FIG. 4. **MS/MS of the disulfide-linked tripeptide for the ion at *m/z* 1172.0.** Details of the fragments observed are shown in Fig. 3*A* and in the *inset* table.

charged ion ($y_{12}Y_4$-H_2O) due to the loss of 18 Da (water) from the precursor ion (Fig. 5). All prominent ions observed, as shown in Fig. 3, can be derived from the sequence of disulfide paired peptides (86–97 and 340–343), confirming the disulfide pattern of Cys^{91}-Cys^{343}. The MS^3 spectrum of the singly charged ion of *m/z* 1750.3 (b_5Y_{10}) is shown in Fig. 6. As predicted, the dominant fragments are derived from the sequence of the disulfide paired peptides 81–85 and 330–339, with sequential loss of N-terminal amino acids from peptide 330–339; confirming the disulfide pattern of Cys^{81}-Cys^{338}.

Analysis of the Tripeptide Complex by Endoproteinase Glu-C Digestion—To further confirm the identity of the disulfide-containing tripeptide complex, fractions of the tryptic digest obtained from the biotin-modified FucT III that eluted between 34 and 38 min from the LC run were collected and digested with Glu-C. The resulting peptides were analyzed by ESI-MS/MS (Fig. 7). As would be predicted from the specificity of Glu-C, ions corresponding to two disulfide-containing peptides (Cys^{91}-Cys^{343} and Cys^{81}-Cys^{338}) were detected. These peptides produced doubly charged ions at *m/z* 769.7 and 995.8, corresponding to calculated values (*m/z* 769.9 and 995.8, due to the cleavage at the C terminus of Glu^{83} found in the peptide containing amino acids 81–97 and Cys^{81} and Cys^{91} (see Fig. 3). The MS/MS analysis (data not shown) of the doubly charged ion at *m/z* 995.8 for the disulfide-containing peptides Cys^{91}-Cys^{342} showed that the dominant product ions are generated from a fragmentation event at the amide bond of Pro^{86} in the peptide containing amino acids 84–97 and Cys^{91}, confirming the disulfide pattern of Cys^{91}-Cys^{341}. The MS/MS spectrum of the doubly charged ion at *m/z* 769.7 (Fig. 7) for the disulfide-containing peptide (Cys^{81}-Cys^{338}) shows that the dominant product ions are the singly charged ions ($Y_{17}Y_n$, *n* = 2–9), that result

	Fragment	Charge	Mass
1	b_{10}	+	442.0
2	y_4	+	462.1
3	$b_{13}Y_4$*	+	709.3
4	$Y_{12}B_2$*	2+	713.9
5	$Y_{12}B_3$*	2+	808.7
6	$Y_{12}B_3$*+H_2O	2+	817.1
7	$Y_{12}Y_4$*-H_2O	2+	871.9
8	Y_8B_3*	+	1288.4
9	Y_7Y_4*	+	1320.8
10	Y_5B_3*	+	1461.6
11	$b_{16}Y_4$*	+	1569.5
12	$Y_{10}B_2$*	+	1587.4

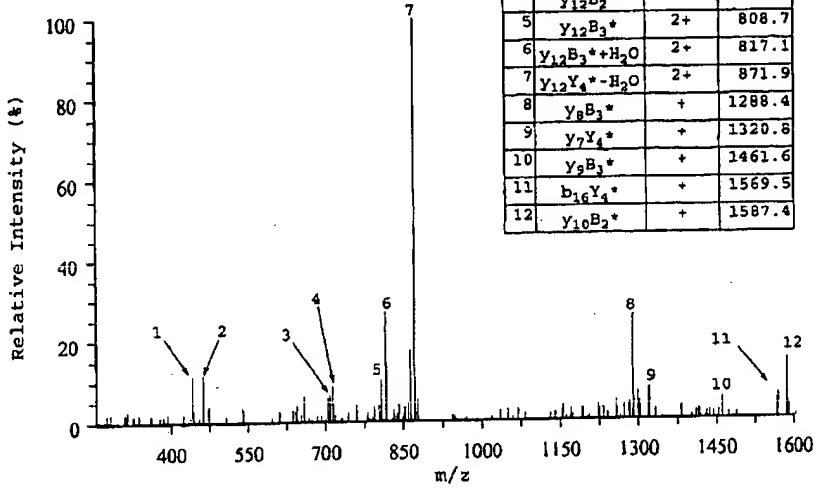


FIG. 5. **MS³ spectrum of doubly charged ion at *m/z* 881.1.** Fragments generated from the $(y_{12}Y_4)^{2+}$ ion, peptide *C* in Fig. 3. $y_{12}Y_4$-H_2O is the dominant ion.

	Fragment	Charge	Mass
1	Y_6+S	+	727.7
2	b_5Y_2	+	797.2
3	b_5Y_3	+	944.1
4	b_5Y_4	+	1058.9
5	b_5Y_5	+	1171.9
6	b_5Y_6	+	1243.2
7	b_5Y_7	+	1430.5
8	b_5Y_8	+	1516.3
9	B_9b_5	+	1604.0
10	b_5Y_{10}-H_2O	+	1732.3
11	M+H	+	1750.3

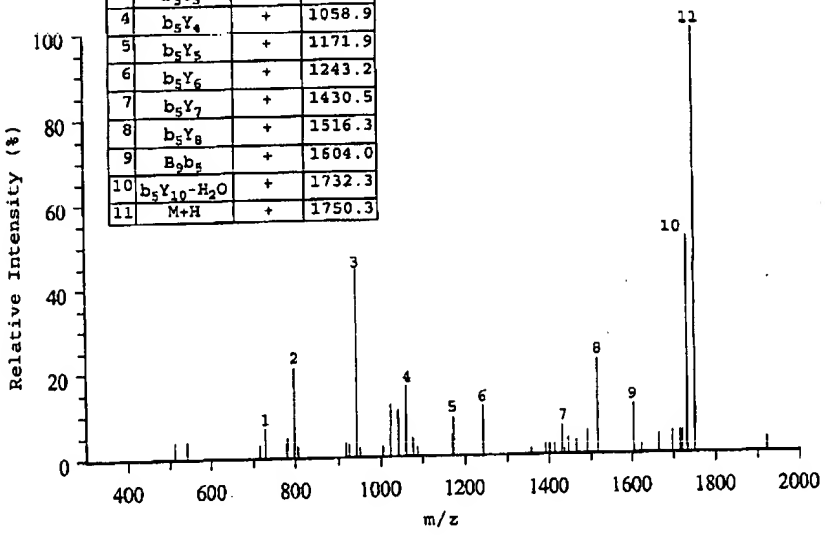
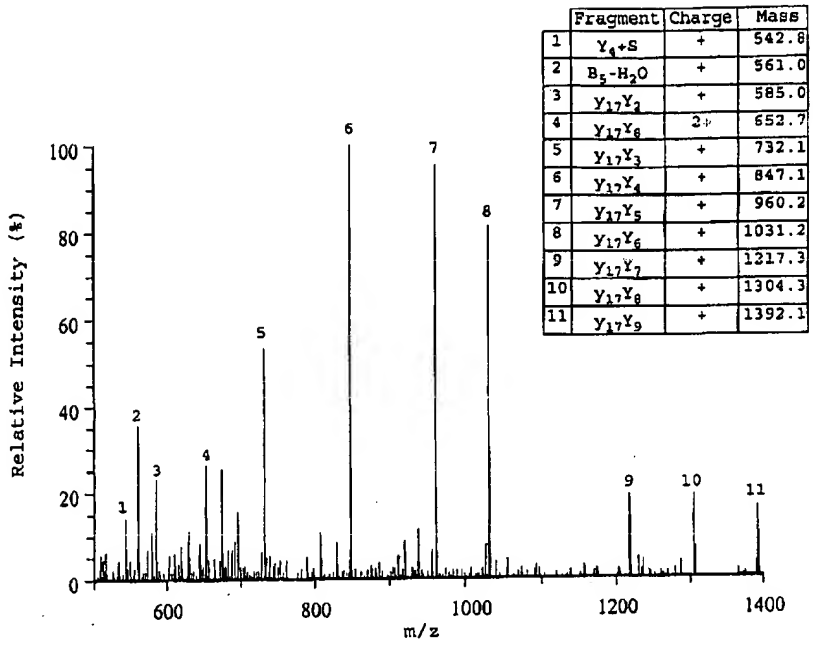


FIG. 6. **MS³ spectrum of the singly charged ion at *m/z* 1750.3.** Fragments generated from the $(b_5Y_{10})^+$ ion, peptide *B* in Fig. 3. The ions b_5Y_n (n = 2–8) are the dominant fragment ions.

from the sequential loss of N-terminal amino acids from peptide 330–339, confirming the disulfide pattern of Cys^{81}-Cys^{338} between peptides 81–83 and 330–339.

Site-directed Mutagenesis of FucT V—To evaluate the functional significance of the highly conserved Cys residues, site-directed mutagenesis studies were carried out using a FucT (FucT V) that is highly homologous to FucT III, differing at only 21 amino acid residues within the catalytic region. A truncated form of the enzyme, containing only the catalytic domain of the enzyme, coupled to a peptide tag corresponding to the protein A, IgG binding domain was used. We have previously established that this tag does not alter the activity or substrate specificity of any of the human FucTs. Furthermore, the tag provides a means of purifying and detecting the enzyme. Finally, the tagged FucT is isolated after secretion into the cell growth medium. Therefore, any expressed form (*i.e.* wild type or mutant) must traverse the entire protein secretory pathway before it is isolated for analysis.

Site-directed mutagenesis was used to change each of the conserved Cys residues in FucT V independently to Ser residues, and a double mutant (Cys^{351} → Ser/Cys^{364} → Ser) was also produced (Fig. 8, *lane 6*). As shown in Fig. 8, each mutant construct produced a protein that could be isolated from the cell growth medium by affinity chromatography. Three of the four single mutants, and the double mutant, produced proteins with a molecular weight similar to that of the wild type FucT V (Fig. 8, *lane 1*). Treatment of the wild type enzyme and these mutants with PNGase F demonstrated that all of these pro-



	Fragment	Charge	Mass
1	Y_4+S	+	542.8
2	B_5-H_2O	+	561.0
3	$y_{17}Y_2$	+	585.0
4	$y_{17}Y_6$	2+	652.7
5	$y_{17}Y_3$	+	732.1
6	$y_{17}Y_4$	+	847.1
7	$y_{17}Y_5$	+	960.2
8	$y_{17}Y_6$	+	1031.2
9	$y_{17}Y_7$	+	1217.3
10	$y_{17}Y_8$	+	1304.3
11	$y_{17}Y_9$	+	1392.1

FIG. 7. **MS/MS of the Glu-C product (see Fig. 3*D*), a doubly charged ion at *m/z* 769.9.** The dominant ions observed are $y_{17}Y_n$ (n = 2–9) using the notation of fragments in Fig. 3*A*.

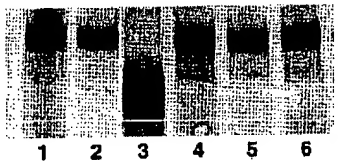


FIG. 8. **Western blot of Cys mutants of human FucT V.** FucT V mutants were prepared by a double PCR mutagenesis technique and expressed as chimeric proteins (catalytic domain of FucT V fused to the IgG binding domain of protein A) in COS-7 cells. The proteins were purified from the growth medium with an IgG resin and chromatographed by SDS-PAGE. *Lane 1*, wild type FucT V; *lane 2*, mutant Cys^{94}; *lane 3*, mutant Cys^{104}; *lane 4*, mutant Cys^{351}; *lane 5*, mutant Cys^{354}; *lane 6*, double mutant Cys^{351}/Cys^{354}.

teins are modified by *N*-linked oligosaccharides (data not shown). The other mutant (Cys^{104} → Ser) produced a broad band on the Western blot that had a faster mobility than the wild type FucT V (Fig. 8, *lane 3*), and some of these bands had a higher gel mobility after treatment with PNGase F. Each protein was assayed for enzyme activity using a range of known acceptor substrates, including the preferred acceptor substrate for FucT V, H-type II. None of the mutant proteins had detectable enzyme activity, even upon prolonged incubation conditions (data not shown). The lack of enzyme activity could result from a variety of reasons (*e.g.* disruption of the protein structure or elimination of an essential functional group that assists in substrate binding or the catalytic mechanism). Therefore, further studies were carried out in an effort to better understand the basis for the lack of enzyme activity.

Photolabeling Experiments—Although the FucT V Cys mutants were enzymatically inactive, it is possible that they may still bind substrate. Since the K_m for binding the nucelotide sugar donor, GDP-Fuc, is significantly lower than that of the acceptor substrate, photolabeling experiments were conducted using the photoaffinity probe ^{125}I-GDP-hexanolamine-ASA (13). Wild type FucT V and FucT V Cys mutants were photolabeled with 12.5 μM ^{125}I-GDP-hexanolamine-ASA (equivalent to 0.5 times the K_i of GDP-hexanolamine with respect to GDP-Fuc; Ref. 13) in the absence and presence of 400 μM GDP-Fuc.

TABLE I
Photoaffinity labeling of wild type FucT V and FucT V Cys mutants with ^{125}I-GDP-hexanolamine-ASA

Enzyme	Specific labeling[a]	Protection[b]
	cpm/100 ng protein	%
FucT V	609	58
C94S	220	36
C104S	26	11
C351S	283	29
C354S	251	42

[a] Values are derived from the difference in extent of labeling of enzyme protein in photolabeling reaction mixtures conducted in the absence and presence of 400 μM GDP-Fuc (specific labeling = cpm incorporated in the absence of GDP-Fuc minus cpm incorporated in the presence of 400 μM GDP-Fuc).

[b] Percentage of protection of photolabeling by GDP-Fuc ([cpm incorporated in the absence of GDP-Fuc − cpm incorporated in the presence of 400 μM GDP-Fuc]/cpm incorporated in the absence of GDP-Fuc] × 100).

After photolysis, the enzyme fractions were separated by SDS-PAGE, and the amount of labeled photoprobe incorporated into protein quantified. The results shown in Table I indicate that a significant level of specific labeling of wild type FucT V occurred (defined as the cpm incorporated into protein in the absence of GDP-Fuc minus the cpm incorporated into protein in parallel reactions in the presence of GDP-Fuc). The extent of protection by GDP-Fuc for wild type FucT V was 58%. The remainder of the cpm incorporated into protein most likely represents nonspecific labeling as the highly reactive nitrene generated upon photolysis reacts with protein sites at random.

The level of specific labeling of the FucT V mutants Cys^{94} → Ser, Cys^{351} → Ser, and Cys^{354} → Ser was lower (35–50% of wild type) when compared with wild type FucT V, but still significant. In addition, substantial protection from photolabeling was observed with excess GDP-Fuc, demonstrating that the photoprobe was competing with GDP-Fuc for binding to each protein. In contrast, there was very little specific labeling observed with the FucT V Cys^{104} → Ser mutant, suggesting this enzyme has lost the ability to bind GDP-Fuc. Since much (>90% estimated from a Coomassie-stained gel) of this protein

was apparently degraded, it was not surprising that there was little incorporation of photoprobe.

DISCUSSION

The existence of a family of FucTs was initially proposed on the basis of differences observed in substrate specificity and sensitivity to inhibition by an amino acid modifying agent (*i.e.* NEM) (see Ref. 17 and references therein). However, it was not possible to establish how the enzymes differed until they were cloned. The predicted amino acid sequence information from their cloning provided several clues that have allowed us and others to begin to identify which amino acids give each enzyme its distinct properties (7, 9, 12, 15, 18–21). For example, among the human FucTs, only some (FucTs III, V, and VI) are inhibited by NEM, and we (15) have established that a free Cys residue accounts for this property in the sensitive enzymes, whereas those that are insensitive (22–24) to NEM have a corresponding Ser (FucT IV) or Thr (FucT VII) residue. More importantly, the identification of the NEM-sensitive Cys residue allowed us to pinpoint an amino acid that lies in or near the binding site for GDP-Fuc. In the current study, we have now chemically established that FucT III does contain a single, free Cys residue at this site. We have also identified a Lys residue that also lies in or near the GDP-Fuc binding site. These two residues occur at a significant distance (equivalent to amino acids Cys^{143} and Lys^{259} in FucT III) from one another in the FucT sequence and therefore, the native fold of the FucTs must bring these residues near one another in space. Furthermore, we have verified that the amino acid sequence predicted from the corresponding DNA is correct and that the only amino acids modified posttranslationally in the yeast expression system are Asn residues at the two predicted *N*-linked sites (Asn^{154} and Asn^{185}). These are the first results available to verify the complete amino acid sequence of any fucosyltransferase, and provide the first information on the location of *N*-linked site, oligosaccharide occupancy for a fucosyltransferase.

We and others have also identified amino acids that affect acceptor substrate specificity and thus are most likely close to one another in the native protein (7, 9, 12, 18, 20). Our previous studies of FucTs III and V have shown that amino acids affecting acceptor substrate specificity lie at the two ends of the catalytic domain of these two enzymes (7, 20). Based on this observation, and the fact that all mammalian FucTs have two sets of conserved Cys residues at the N and C termini of their catalytic domain, we (see discussion of Ref. 7) proposed that disulfide bonds between one or both pairs of the highly conserved Cys residues are responsible for bringing the two ends of the catalytic domain close together in the native protein, and thus bringing the residues identified as being important for acceptor substrate specificity together. The results reported in this study verify that this hypothesis is true. Furthermore, the results demonstrate that both pairs of conserved Cys residues are involved in disulfide bonds and that Cys^{91}-Cys^{341} and Cys^{81}-Cys^{338} of FucT III are bonded together. The latter point was unequivocally established by a combination of MS experiments including MS^3 analyses of the disulfide-linked tripeptide tryptic peptide isolated from nonreduced FucT III, and MS/MS analyses of the GluC products obtained from the disulfide-linked tryptic peptide. These results establish the maximum distance (not more than 35 Å) that amino acids we had previously demonstrated to affect acceptor substrate specificity (His^{73}-Ile^{74} and Asp^{336} in FucT III, separated by more than 250 amino acid residues in the linear sequence) can be from one another in space when the disulfide bonds are formed.

We[3] have recently demonstrated that FucT VII has a different disulfide bond pattern than that reported here for FucT III. This protein contains the four highly conserved Cys residues found in other FucTs (Fig. 1) plus two additional, closely spaced Cys residues (Cys^{211} and Cys^{214}) in the middle of its catalytic domain. Our results demonstrate that all six Cys residues form disulfide bonds and that each closely spaced pair (*i.e.* Cys^{68} to Cys^{76}, Cys^{211} and Cys^{214}, and Cys^{318} to Cys^{321}) of Cys residues are linked together. This results in a protein containing three short loops, in contrast to the large, single loop pattern that occurs in FucT III. Although we currently do not know whether the Cys residues in other FucTs are involved in disulfide bonds, it seems reasonable to expect that FucT V and VI would have the same disulfide bond pattern as FucT III since these enzymes have very highly conserved amino acid sequences, especially within their catalytic domain. Furthermore, we (12) and Lowe and co-workers (9) have demonstrated that it is possible to swap segments of the corresponding amino acid sequences of these proteins and create active domain swap mutants (*i.e.* swapping segments that do not contain all of the conserved Cys residues). Thus, it seems likely, although still unproven, that FucTs III, V, and VI would share a common disulfide bond pattern. Since human FucT IV and FucTs expressed in other species share ≤50% sequence homology with FucT III and VII, it is difficult to predict whether they will share the disulfide bond pattern of FucT III or VII, or have a distinct pattern.

The fact that both FucT III and VII bind GDP-Fuc but do not share a common disulfide bonding pattern suggests that the disulfide bonding pattern in these enzymes is not required for or involved in the formation of the GDP-Fuc binding pocket. The results obtained from our photolabeling experiments are consistent with this since the Cys mutants could still be labeled with the GDP-photo probe and labeling was blocked by GDP-Fuc.

Even though the mutant enzymes can bind GDP-Fuc, they are inactive. This may indicate that the acceptor substrate binding pocket for FucT V (and by analogy FucT III and VI) is dependent on the proper formation of disulfide bonds. Since these enzymes have a broader acceptor substrate specificity than FucT VII, one would anticipate that their acceptor substrate binding pockets would differ from that of FucT VII (see Ref. 11 and references therein; see also Refs. 22 and 23). The difference in the disulfide bond pattern of these FucTs could be critical for the formation of these distinct acceptor substrate binding sites. Further studies will be necessary to establish whether this is the case.

Another interesting observation from the mutagenesis studies is that only one of the mutants, FucT V $Cys^{104} \rightarrow$ Ser, gave a Western blot pattern that differed significantly from the wild type enzyme. This protein was always found to be substantially degraded, whereas all of the other mutants, including the double mutant, gave a pattern essentially identical to the wild type enzyme. It is not clear why the conversion of one of the highly conserved Cys residues in the FucTs sequence should lead to a misfolded protein that is degraded more rapidly than the other mutants or the wild type protein. However, it is interesting to point out that this Cys residue lies near a potential *N*-linked glycosylation site (not one of those conserved in FucT III); a site that we have previously shown is not glycosylated in wild type FucT V (7). Thus, it is possible that substitution of a Ser residue for the Cys residue closest to this *N*-linked site causes some alteration that allows *N*-linked glycosylation to occur and

[3] Sherwood, A. L., Davis, W. C., Ho, S., Macher, B. A., Stroud, M. R., Upchurch, D. A., and Holmes, E. H. (2000) *Biochem. Biophys. Res. Commun.*, in press.

[3] T. de Vries, T-Y., Yen, R. K. Joshi, J. Storm, D. H. van den Eijnden, R. M. A. Knegtel, H. Bunschoten, D. H. Joziasse, and B. A. Macher, submitted for publication.

prohibits proper folding of the protein. Further studies are required to establish the actual cause of the misfolding and degradation of the FucT V $Cys^{104} \rightarrow Ser$ mutant.

Comparisons of FucT amino acid sequences from several species have suggested that the α1,3 fucosyltransferase family of enzymes were derived from a common ancestral gene, and that the enzymes found today have evolved by gene duplication and divergence (1–3). Gene duplication of a common ancestral gene originated the leukocyte (FucT VII), myeloid (FucT IV), and Lewis (FucT III, V, and VI) subfamilies. Based on the results reported in this study, and those to be reported elsewhere on FucT VII's disulfide bond pattern, not only have the amino acid sequences of the FucTs diverged during evolution but, so have the pattern of their disulfide bonds. It will be interesting to determine the disulfide bond pattern of the other members of the FucT families and incorporate the resulting information into an assessment of the evolution of this family of enzymes. It is interesting to note that the FucT reported by DeBose-Boyd *et al.* (6) from *C. elegans* does not contain the N-terminal Cys residues conserved in other species and, therefore, would not be capable of forming the disulfide bond pattern found for FucT III, but could form a disulfide bond equivalent to that found in FucT VII, between the highly conserved Cys residues near the C terminus of the catalytic domain. It will be interesting to determine if this is the case or if FucTs from lower order organisms have a completely different pattern.

REFERENCES

1. Breton, C., Oriol, R., and Imberty, A. (1998) *Glycobiology* **8**, 87–94
2. Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., and Breton, C. (1999) *Glycobiology* **9**, 323–334
3. Oulmouden, A., Wierinckx, A., Petit, J. M., Costache, M., Palcic, M. M., Mollicone, R., Oriol, R., and Julien, R. (1997) *J. Biol. Chem.* **272**, 8764–8773
4. Leiter, H., Mucha, J., Staudacher, E., Grimm, R., Glössl, J., and Altmann, F. (1999) *J. Biol. Chem.* **274**, 21830–21839
5. Kageyama, N., Natsuka, S., and Hase, S. (1999) *J. Biochem. (Tokyo)* **125**, 838–845
6. DeBose-Boyd, R. A., Nyame, A. K., and Cummings, R. D. (1998) *Glycobiology* **8**, 905–917
7. Nguyen, A. T., Holmes, E. H., Whitaker, J. M., Ho, S., Shetterly, S., and Macher, B. A. (1998) *J. Biol. Chem.* **273**, 25244–25249
8. Weston, B. W., Smith, P. L., Kelly, R. J., and Lowe, J. B. (1992) *J. Biol. Chem.* **267**, 24575–24584
9. Legault, D. J., Kelly, R. J., Natsuka, Y., and Lowe, J. B. (1995) *J. Biol. Chem.* **270**, 20987–20996
10. Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications*, (Innis, M. A., Gelfand, D. H., and Sninsky, J. J., eds) pp. 177–183, Academic Press, New York
11. de Vries, T., Srnka, C. A., Palcic, M. M., Swiedler, S. J., van den Eijnden, D. H., and Macher, B. A. (1995) *J. Biol. Chem.* **270**, 8712–8722
12. Xu, Z., Vo, L., and Macher, B. A. (1996) *J. Biol. Chem.* **271**, 8818–8823
13. Holmes, E. H. (1990) *J. Biol. Chem.* **265**, 13150–13156
14. Gallet, P. H., Vaujour, H., Petit, J. M., Maftah, A., Oulmouden, A., Oriol, R., Le Narvor, C., Guilloton, M., and Julien, R. (1998) *Glycobiology* **8**, 919–925
15. Holmes, E. H., Xu, Z., Sherwood, A. L., and Macher, B. A. (1995) *J. Biol. Chem.* **270**, 8145–8151
16. Loo, J. A., Edmonds, C. G., and Smith, R. D. (1993) *Anal. Chem.* **65**, 425–438
17. Macher, B. A., Holmes, E. H., Swiedler, S. J. Stults, C. L. M., and Srnka, C. A. (1991) *Glycobiology* **1**, 577–584
18. Dupuy, F., Petit, J. M., Mollicone, R., Oriol, R., Julien, R., and Maftah, A., (1999) *J. Biol. Chem.* **274**, 12257–12262
19. Sherwood, A. L., Nguyen, A. T. Whitaker, J. M., Macher, B. A., and Holmes, E. H., (1998) *J. Biol. Chem.* **273**, 25256–25260
20. Vo, L., Lee, S., Marcinko, M. C., Holmes, E. H., and Macher, B. A. (1998) *J. Biol. Chem.* **273**, 25250–25255
21. Kaneko, M., Kudo, T., Iwasaki, H., Ikehara, Y., Nishihara, S., Nakagawa, S., Sasaki, K., Shiina, T., Inoko, H., Saitou, N., and Narimatsu, H. (1999) *FEBS Lett.* **452**, 237–242
22. Lowe, J. B., Kukowska-Latalo, J. F., Nair, R. P., Larsen, R. D., Marks, R. M., Macher, B. A., Kelly, R. J., and Ernst, L. K. (1991) *J. Biol. Chem.* **266**, 17467–17477
23. Natsuka, S., Gersten, K. M., Zenita, K., Kannagi, R., and Lowe, J. B. (1994) *J. Biol. Chem.* **269**, 16789–16794
24. Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., Hanai, N., and Nishi, T. (1994) *J. Biol. Chem.* **269**, 14730–14737

PATENT
Attorney Docket No. 040853-01-5108-US
Client Ref. No.: NEO00073

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation Number: 1113
Robert Bayer	Examiner: Rao, Manjunath
Application No.: 09/855,320	Technology Center/Art Unit: 1652
Filed: May 14, 2001	DECLARATION OF DAVID ZOPF UNDER 37 C.F.R. §1.132
For: IN VITRO MODIFICATION OF GLYCOSYLATION PATTERNS OF RECOMBINANT GLYCOPEPTIDES	
Customer No.: 43850	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, David A. Zopf, M.D. declare as follows:

1. I am Executive Vice President and Chief Scientific Officer of Neose Technologies, Inc. Prior to joining Neose, I was Vice President and Chief Operating Officer of BioCarb, Inc. In addition, I am a former editorial board member of *Archives of Biochemistry and Biophysics*. My *Curriculum Vitae* is attached.

2. I am submitting this declaration to testify for the initial expert skepticism and subsequent commercial success associated with the *in vitro* fucosylation methods provided by the present application.

3. Attached as Exhibit E is a copy of a letter from Professor Dr. James E. Bailey, Institute of Biotechnology, ETH-Zürich, CH-8093 Zurich, Switzerland. In my opinion, the attached letter is an representative example of expert skepticism in the field towards *in vitro* fucosylation methods, especially large scale production provided by the present application.

4. In general, the letter expresses disbelief that an *in vitro* glycosylation process, such as the presently claimed fucosylation process, is commercially viable. Dr. Bailey states that "the process complications and costs associated with producing and utilizing a glycosyltransferase and donor substrate make the exogenous manipulation of glycosylation far less attractive than engineering the cells to maximize the production of the desired glycoform." Dr. Bailey continues by saying, "[f]ailure of much simpler cofactor-requiring enzyme catalyzed reactions to gain industrial success in competition with whole-cell biocatalysts speaks very strongly in my opinion *against* the competitive prospects of *in vitro* remodeling of glycosylation." [Emphasis added].

5. In addition to the foregoing skepticism by experts in the field, the technology, both for *in vitro* glycosylation in general and for *in vitro* fucosylation in particular, has enjoyed commercial success.

6. In my capacity as Executive Vice President and Chief Scientific Officer (and in my previous capacity as Vice President of New Product Development) of Neose, I have directly participated in negotiating agreements with several companies in order to assess the feasibility of our GlycoAdvance fucosylation technology (*in vitro* fucosylation) with recombinant therapeutic glycoproteins in development. Although most of these agreements are subject to non-disclosure agreements which prevent me from disclosing our studies, I am at liberty to disclose two licensing agreements involving our GlycoAdvance fucosylation technology.

7. The first GlycoAdvance fucosylation agreement was with Wyeth/Ayerst Laboratories, the pharmaceutical division of American Home Products. Under this agreement, we employed our GlycoAdvance fucosylation technology for an improved production system for Wyeth's inflammation/thrombosis P selectin antagonist, rPSGL-Ig. We have successfully provided highly fucosylated peptides of Wyeth using the *in vitro* fucosylation methods provided by the present invention. Due to disappointing Phase II clinical trial results, however, the clinical development of this compound was suspended. Please note that Neose's contribution to this technology was successful, and the compound development was suspended for other reasons

that were unrelated to GlycoAdvance. Press releases from Neose announcing both the initiation and suspension of the Wyeth relationship are included as Exhibit F and Exhibit G, respectively.

8. The second GlycoAdvance fucosylation agreement was with Avant Immunotherapeutics. Under this agreement, we employed our GlycoAdvance fucosylation technology for an improved production system for Avant's complement system inhibitor, sCR1-sLe^x. This collaboration was successful, meaning that our GlycoAdvance fucosylation technology was able to consistently fucosylate Avant's target molecule. The results of this collaboration were published this year (Thomas, L.J. *et al.*, *Glycobiology* **14(10)**: 883-893 (2004)). This paper is included as Exhibit B.

9. These two specific *in vitro* fucosylation examples are part of the larger success of Neose's *in vitro* glycosylation strategies. In my capacity as Executive Vice President and Chief Scientific Officer (and in my previous capacity as Vice President of New Product Development) of Neose, I have been directly involved in negotiating agreements with more than 20 companies in order to assess the feasibility of *in vitro* glycosylation technology for recombinant therapeutic glycoproteins in development. All feasibility studies completed to date have been successful. Many of these successful feasibility studies have led to ongoing negotiations for commercial licenses to the technology for large-scale manufacture of human glycoprotein therapeutics. In addition, the present technology is being employed as an essential part of ongoing collaborative research and development agreements with other companies to develop commercial manufacturing methods for cancer vaccines and treatments for neurological diseases. Press releases relating to this are noted in Exhibit H.

10. In my opinion, this commercial success is directly related to the innovative *in vitro* fucosylation methods provided by the present invention and also reflects the long felt needs and failure by others in the field.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so

PATENT

made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 7 Dec 2004

David A. Zopf, M.D.

CURRICULUM VITAE

David A. Zopf, M.D.

Citizenship	United States
Social Security #	493-44-7185
Present Address	Neose Technologies, Inc. 102 Witmer Road Horsham, PA 19044 Phone (215) 441 5890 Fax (215) 441 5896

Education

1964 - A.B., Washington University, St. Louis, MO (Zoology Major)
1969 - M.D., Washington University School of Medicine, St. Louis, MO

Board Certification — Anatomic Pathology, 1976

Brief Chronology of Employment

2000 – present	Vice President, New Product Development Neose Technologies, Inc., Horsham,
1992- 2000	Vice President, Drug Development Neose Technologies, Inc., Horsham, PA
1988-1991	Vice President and Chief Operating Officer, BioCarb Inc, Gaithersburg, MD
1982-1988	Chief, Section on Biochemical Pathology Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1977-1981	Expert Consultant, Laboratory of Pathology, Division Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD
1974-1977	Senior Staff Fellow, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD
1971-1974	Clinical Associate, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD

1970-1971	Resident, Anatomic Pathology, Department of Pathology, University of Colorado School of Medicine, Denver, CO
1969-1970	Resident, Anatomic Pathology, Department of Pathology, New York University, Bellevue Medical Hospital, NY
Military Service	Active duty July 1971-1974; 1981-1988 Commissioned Corps, U.S. Public Health Service, National Institutes of Health, Bethesda, MD
Societies	American Association for the Advancement of Science Society for Glycobiology Pluto Club (emeritus) American Association of Pathologists American Society of Biochemistry and Molecular Biology American Chemical Society
Editorial Boards	Archives Biochemistry and Biophysics, Section on Immunochemistry and Complex Carbohydrates Experimental Pathology Glycoconjugate Journal (1984 - 1991)
Research Interests	Immunochemistry of complex carbohydrates Biochemistry of human blood groups Immune responsiveness to complex carbohydrates Structure, function, and biosynthesis of tumor-associated antigens Affinity methods for analysis of complex carbohydrates Development of oligosaccharide anti-infective drugs

BIBLIOGRAPHY

1. Kinsky, S.L., Luse, S.A., Zopf, D.A., van Deenen, L.L.M., and Haxby, J.: Interaction of filipin and derivatives with erythrocyte membranes and lipid dispersions: electron microscopic observations. Biochem. Biophys. Acta 135: 844-861, 1967.

2. Kinsky, S.C., Haxby, J.A., Zopf, D.A., Alving, C.R., and Kinsky, C.B.: Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. Biochemistry 8: 4149-4158, 1969.

3. Zopf, D.A.: Electron microscopic and osmotic studies on the effect of polyene antibiotics upon natural and artificial membranes. Borden Award Thesis, Washington University School of Medicine, 1969.

4. Zopf, D.A., and Ginsburg, V.: Carbohydrate antigens of cell surfaces. In Lee, E.Y.C. and Smith, E.E. (Eds.): Biology and Chemistry of Eucaryotic Cell Surfaces. New York, Academic Press, 1974, vol. 7, 259-271.

5. Zopf, D.A., and Ginsburg, V.: Solubilization and expression of cell surface components. In Kahan, B.D. and Reisfeld, R.A. (Eds.): The Cell Surfaces. Immunological and Chemical Approaches. New York, Plenum Press, 1974, 51-57. (Adv. Exp. Med. Biol., Vol. 51).

6. Zopf, D.A. and Ginsburg, V.: Preparation of precipitating antigens by coupling oligosaccharides to polylysine. Arch. Biochem. Biophys. 167: 345-350, 1975.

7. Jeffrey, A.M., Zopf, D.A., and Ginsburg, V.: Affinity chromatography of carbohydrate-specific immunoglobulins: coupling of oligosaccharide to Sepharose. Biochem. Biophys. Res. Commun. 62: 608-613, 1975.

8. Zopf, D.A., Ginsburg, A., and Ginsburg, V.: Goat antibody directed against a human Leb blood group hapten, lacto-N-difucohexaose I. J. Immunol. 115: 1525-1529, 1979

9. Ginsburg, V., Zopf, D.A., Yamashita, K., and Kobata, A.: Oligosaccharides of human milk: isolation of a new pentasaccharide, lacto-N-fucopentaose V. Arch. Biochem. Biophys. 175: 565-568, 1976.

10. Tsai, C.M., Zopf, D.A., Yu, R.K., Wistar, R., Jr., and Ginsburg, V.: A human cold agglutinin specific for lacto-N-neotetraose. J. Immunol. 117: 717-721, 1976.

11. Tsai, C.-M., Zopf, D.A., Yu, R.L., Wistar, R., Jr., and Ginsburg,V. A Waldenstrom macroglobulin that is both a cold agglutinin and a cryoglobulin because it binds N-acetylneuraminosyl residues. Proc. Natl. Acad. Sci. USA 74: 4591-4594, 1977.

12. Zopf, D.A., Tsai, C.-M., and Ginsburg, V. Studies on the carbohydrate receptors of cold agglutinins using synthetic antigens. In Mohn, J.F., Plunkett, R.W., Cunningham, R.K., and Lambert, R.M. (Eds.): Human Blood Groups. Basel,Switzerland, S. Karger AG, 1977, pp. 172-178.

13. Zopf, D.A., Tsai, C.-M., and Ginsburg, V. Antibodies against oligosaccharides coupled to proteins: Characterization of carbohydrate specificity by radioimmune assay. Arch. Biochem. Biophys. 185: 61-71, 1978.

14. Tsai, C.-M., Zopf, D.A., and Ginsburg, V. The molecular basis for cold agglutination: Effect of receptor density upon thermal amplitude of a cold agglutinin. Biochem. Biophys. Res. Commun.80: 905-910, 1978.

15. Smith, D.F., Zopf, D.A., and Ginsburg, V. Fractionation of sialyl oligosaccharides of human milk by ion-exchange chromatography. Anal. Biochem. 85: 602-608, 1978.

16. Zopf, D.A., Tsai, C.-M., and Ginsburg, V. Carbohydrate antigens: coupling of oligosaccharide-phenethylamine derivatives to edestin by diazotization and characterization of antibody specificity by radioimmunoassay. In Ginsburg, V.(Ed.): Methods in Enzymology. New York, Academic Press, 1978, Vol. L, pp. 163-169.

17. Zopf, D.A., Drzeniek, Z., Smith, D.F., Tsai, C.-M., and Ginsburg, V. Carbohydrate antigens: affinity purification of antibodies using oligosaccharide-phenethylamine derivatives coupled to Sepharose. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1978, Vol. L, pp. 171-175.

18. Smith, D.F., Zopf, D.A., and Ginsburg, V. Carbohydrate antigens: Coupling of oligosaccharide-phenethylamine-isothiocyanate derivatives to bovine serum albumin. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1978, Vol. L, pp. 169-171.

19. Smith, D.F., Zopf, D.A., Ginsburg, V. Sialyl oligosaccharides from milk. In Ginsburg, V.(Ed.): Methods in Enzymology. New York, Academic Press, 1978, Vol. L, pp, 221-226.

20. Zopf, D.A., Tsai, C-M., and Ginsburg, V. Antibodies against oligosaccharides. In Goldstein, I.J., (Ed.): Carbohydrate-Protein Interaction. Washington, D.C., American Chemical Society, 1979, pp. 90-101.

21. Zopf, D.A., Ginsburg, V., Hallgren, P., Johnson, A.C., Lindberg, B.S, and Lundblad, A. Determination of Le^b-active oligosaccharides, in urine of pregnant and lactating women by radioimmunoassay. Eur. J. Biochem. 93: 431-435, 1979.

22. Rohr, T.E., Smith, D.F., Zopf, D.A., and Ginsburg, V. Le^b-active glycolipid in human plasma: measurement by radioimmunoassay. Arch. Biochem. Biophys. 199: 265-269, 1980.

23. Ginsburg, V., McGinniss, M.M., and Zopf, D.A. Biochemical basis for some blood groups. In Sandler, S.G., Nusbacher, J., and Schanfield, M.S., (Eds.): Immunobiology of the Erythrocyte. New York, Alan R. Liss, Inc., 1980, pp. 45-53.

24. Warner, C.V, Smith, D.F., Zopf, D.A., and Ginsburg, V. A human Le(a-b+) blood group agglutinin prepared by polymerizing goat anti-lacto-N-difucohexaose I IgG. Molec. Immunol. 17:1163-1166, 1980.

25. Hammar, L., Mansson, S., Rohr, T., Chester, M.A., Ginsburg, V., Lundblad, A., and Zopf, D.A.: Decreased expression of the Le^b antigen during pregnancy: Determination of erythrocyte agglutinability and Le^b-active glycolipid in serum. Vox Sang. 40: 27-33, 1981.

26. Zopf, D.A, Levinson, R.E., and Lundblad, A. Determination of a glucose-containing tetrasaccharide in urine by radioimmunoassay. J. Immunol. Meth. 48: 109-119, 1982.

27. Stein, K.E., Zopf, D.A., Johnson, B.M., Miller, C.B., and Paul, W.E. The immune response to an isomaltohexosyl-protein conjugate, a thymus-dependent analogue of (α1-6)dextran. J. Immunol. 128: 1350-1354, 1982.

28. Nilsson, B., and Zopf, D.A. Gas chromatography and mass spectrometry of hexosamine-containing oligosaccharide alditols as their permethylated, N-trifluoroacetyl derivatives. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1982, Vol. 83, pp. 46-58.

29. Magnani, J.L., Nilsson, B., Brockhaus, M., Zopf, D.A., Steplewski, Z., Koprowski, H., and Ginsburg, V. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. J. Biol. Chem. 257: 14365-14369, 1982.

30. Stein, K.E., Zopf, D.A., Miller, C.B., Johnson, B.M., Mongini,P.K.A., Ahmed, A., and Paul, W.E. The immune response to a thymus dependent form of B512 dextran requires the presence of lyb5+ lymphocytes. J. Exp. Med. 157: 657-666, 1983.

31. Nilsson, B., and Zopf, D.A.: Oligosaccharides released from glycolipids by trifluoroacetolysis can be analyzed by gas chromatography/mass spectrometry. Arch. Biochem. Biophys. 222: 628-648, 1983.

32. Ugorski, M., Seder, A., Lundblad, A., and Zopf, D. Studies on the metabolic origin of a glucose-containing tetrasaccharide in human urine. J. Exp. Pathol. 1: 27-38, 1983.

33. Rao, A.S., Kabat, E.A., Whittaker, N.F., Nilsson B., Zopf, D.A., and Nimmich,W. Isolation and characterization of a 3,4-pyruvic acetylated-D-galactose-containing oligosaccharide obtained from the capsular polysaccharide of Klebsiella K33 by Smith degradation. Carbohydr. Res. 116: 271-276, 1983.

34. Rao, A.S., Kabat, E.A., Nilsson, B., Zopf, D.A., and Nimmich, W.: Isolation and characterization of a 4-0-[3,4-0-(1-carboxy-ethylidene)-α-D-galactopyranosyl] erythritol from Klebsiella K33 polysaccharide. Carbohydr. Res. 121: 205-209, 1983.

35. Chester, M.A., Lundblad, A., Hager, A., Sjöblad, S., Loonen, C.,Tager, J.M., and Zopf, D.A. Increased urinary excretion of a glycogen-derived tetrasaccharide in heterozygotes with glycogen storage disease type II and III. Lancet No. 8331: 994-995, 1983.

36. Ugorski, M., Nilsson, B., Schroer, K., Cashel, J.A., and Zopf, D. GC/MS analysis of oligosaccharides from neutral glycosphingolipids of murine B cell hybridoma. J. Biol. Chem. 259: 481-486, 1984.

37. Messeter, L., Brodin, T., Chester, M.A., Karlsson, K.-A., Zopf, D.A., and Lundblad, A. Immunochemical characterization of a mono-clonal anti-Leb blood grouping reagent. Vox Sang. 46: 66-74, 1984.

38. Lundblad, A., Schroer, K., and Zopf, D. Radioimmunoassay of a glucose-containing tetrasaccharide using a monoclonal antibody. J. Immunol. Meth. 68:217-226, 1984.

39. Lundblad, A., Schroer, K., and Zopf, D.: Affinity purification of a glucose-containing oligosaccharide using a monoclonal antibody. J. Immunol. Meth. 68:227-234, 1984.

40. Wu, A.M., Kabat, E.A., Nilsson, B., Zopf, D.A., Gruezo, F.G., and Liao, J. Immunochemical studies on blood groups. LXXI. Purification and characterization of radioactive [3H] reduced di- to hexa-saccharides produced by alkaline β-elimination-borohydride [3H] reduction of Smith degraded blood group A active glycoproteins. J. Biol. Chem. 259: 7178-7186, 1984.

41. McGinniss, M.H., Wasniowska, K., Zopf, D.A., Straus, S.E., and Reichert, C.M. An erythrocyte Pr auto-antibody with sialoglycoprotein specificity in a patient with purine nucleoside phosphorylase deficiency. Transfusion 25: 131-136, 1985.

42. Wasniowska, K., Reichert, C.M., McGinniss, M.H., Schroer, K.R., Zopf, D.A. Monoclonal antibodies specific for the blood group N determinant. Glycoconjugate J. 2: 163-176, 1985.

43. Hansson, G.C., and Zopf, D.A. Biosynthesis of the cancer-associated sialyl-Le^a antigen. J. Biol. Chem. 260: 9288-9392, 1985.

44. Kumlien, J., Frejd., T., Magnusson, G., Zopf, D.A., and Lundblad, A. A new non-equilibrium enzyme linked immunosorbent assay for a glycogen-derived urinary tetrasaccharide. Glycoconjugate J. 3: 85-94, 1986.

45. Dakour, J., Hansson, G.C., Lundblad, A., and Zopf, D.A. Separation and partial sequence analysis of blood group A-active oligosaccharides by affinity chromatography using monoclonal antibodies. Arch. Biochem. Biophys. 248: 677-683, 1986.

46. Nilsson, B., Rosen, S.W., Weintraub, B.D., and Zopf, D.A.: Differences in the carbohydrate moieties of the common subunits of human chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, and thyrotropin: Preliminary structural inferences from direct methylation analysis. Endocrinology 119: 2737-2743, 1986.

47. Dakour, J., Lundblad, A., and Zopf, D.A. Separation of blood group A-active oligosaccharides by high-pressure liquid affinity chromatography using a monoclonal antibody bound to concanavalin A silica. Anal. Biochem. 161: 140-143, 1987.

48. Mato, J.M., Kelly, K.L., Alber, A., Jarett, L., Corkey, B.E., Cashel, J.A., and Zopf, D.A. Partial structure of an insulin-sensitive glycophospholipid. Biochem. Biophys. Res. Commun. 146: 764-770, 1987.

49. Brodin, T., Chester, M.A., Karlsson, K.-A., Messeter, L., Zopf, D.A., and Lundblad, A. A monoclonal antibody that recognizes both Leb and Y antigens. Glycoconjugate J. 4: 399-406, 1987.

50. Zopf, D.A., Schroer, K., Hansson, G., Dakour, J., and Lundblad, A.: Affinity purification of oligosaccharides using monoclonal antibodies. Meth. Enzymol. 138: 307-313, 1987.

51. Hansson, G.C., Wasniowski, K., Rock, J.A., Ness, P.M., Kickler, T.S., Shirey, R.S., Neibyl, J.R., and Zopf, D.A.: The glycosphingolipid composition of the placenta of a blood group P fetus delivered by a blood group pk-woman and analysis of the anti-globoside antibodies found in the maternal serum. Arch. Biochem.Biophys. 260: 168-176, 1988.

52. Wasniowska, K., Schroer, K.R., McGinniss, M., Reichert, C., and Zopf, D.A. Monoclonal antibodies against glycophorin A. Hyridoma 7: 49-54, 1988.

53. Mårtensson, S., Due, C., Nilsson, B., Påhlsson, P., Ericsson, H., Zopf, D.A., Olsson, L., and Lundblad, A.: A carbohydrate epitope associated with human squamous lung cancer. Cancer Res. 48: 2125-2131, 1988.

54. Dakour, J., Lundblad, A., and Zopf, D.A. Detection and isolation of oligosaccharides with Lea and Leb blood group activities by affinity chromatography using monoclonal antibodies. Arch.Biochem. Biophys. 264: 203-213, 1988.

55. Ohlson, S., Lundblad, A., and Zopf, D.A. Novel approach to affinity chromatography using "weak" monoclonal antibodies. Anal.Biochem. 169: 204-208, 1988.

56. Zopf, D.A., and Hansson, G. The chemical basis for expression of the sialyl-Le^a antigen. In Wu, A. (Ed.): Molecular Immunology ofComplex Carbohydrates. New York, Plenum Press, pp. 657, 1988.

57. Kumlien, J., Chester, M.A., Lindberg, B.S., Pizzo, P., Zopf, D.A. and Lundblad, A. Urinary excretion of a glucose-containing tetrasaccharide: a parameter for increased degradation of glycogen. Clin. Chim. Acta. 176: 39-48, 1988.

58. Kumlien, J., Grönberg, G., Nilsson, B., Månsson, O., Zopf, D.A., and Lundblad, A.: Structural and immunochemical analysis of three limit dextrin oligosaccharides. Arch. Biochem. Biophys. 269: 678-699, 1989.

59. Wang, W.T., and Zopf, D.A. Liquid ion-exchange chromatography of milk oligosaccharides using a pulsed amperometric detector. Carbohydr. Res. 189: 1-11, 1989.

60. Kumlien, J., Andren-Sandberg, A., Zopf, D., and Lundblad, A. Determination of a glucose-containing tetrasaccharide in urine of patients with acute pancreatitis. International Journal of Pancreatology. 4: 139-147, 1989.

61. Wang, W.T., Kumlien, J., Ohlson, S., Lundblad, A., and Zopf, D. Analysis of a glucose-containing tetrasaccharide by high-performance liquid affinity chromatography. Anal. Biochemistry. 182: 48-53, 1989.

62. Zopf, D.A., Ohlson, S., Dakour, J., Wang, W., and Lundblad, A. Analysis and Purification of Oligosaccharides by High-Performance Liquid Affinity Chromatography. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1989, Vol. 179, pp. 55-64.

63. Zopf, D.A., and Ohlson, S. Weak-affinity chromatography. Nature. 346: No. 6279, 87-88, 1990.

64. Wang, W.T., Safar, J., and Zopf, D. Analysis of inositol by high-performance liquid chromatography. Anal. Biochemistry. 188: 432-435, 1990.

65. Wang, W.T., Erlansson, K., Lindh, F., Lundgren, T., and Zopf, D.A. High-performance liquid chromatography of sialic acid-containing oligosaccharides and acidic monosaccharides. Anal. Biochemistry. 190: 182-187, 1990.

66. Safar, J., Wang, W., Padgett, M.P., Ceroni, M., Piccardo, P., Zopf, D.A., and Gajdusek, D.C. Molecular mass, biochemical composition and physiochemical behavior of the infectious form of the scrapie precusor protein monomer. Proc. Natl. Acad. Sci. 87: 6373-6377, 1990.

67. Uppugunduri, S., Dakour, J., Kallin, E., Norberg, T., Zopf, D.A., and Lundblad, A. Affinity purification of monoclonal antibodies using a bifunctional oligosaccharide hapten. Glycoconjugate J. 8: 102-107, 1991.

68. Wang, W.T., Lundgren, T., Lindh, F., Nilsson, B., Grönberg, G., Brown, J.P., Mentzer-Dibert, H., and Zopf, D.A. Isolation of two novel sialyl-Lewis X-active oligosaccharides by high performance liquid chromatography using monoclonal Onc-M26. Arch. Biochem. Biophys. 292: 433-441, 1992.

69. Dakour, J., Zopf, D., and Lundblad, A.: Assay of alpha-1,3 N-Acetyl-D-Galactosaminyl Transferase by Affinity Chromatography. Anal. Biochemistry. 204: 210-214, 1992.

70. Ohlson, S. and Zopf, D. Weak affinity chromatography. in Kline, T., (ed) Handbook of Affinity Chromatography, Marcel Dekker, Inc., New York, 1993.

71. Ohlson, S., and Zopf, D. Weak affinity chromatography. in Ngo T.T. (ed) Molecular Interactions in Bioseparations, pp 15-25, Plenum Press, NY, 1993

72. Zopf, D.A. and Wang, W-T.: Purification of oligosaccharide antigens by weak affinity chromatography. In Garegg, P.J. (ed) Carbohydrate Antigens, ACS Symposium Series, American Chemical Society, Washington DC, 1993.

73. Roth, S. and Zopf, D.: Oligoaccharide pharmaceuticals. In Weiner, D.B. and Williams, W.V. (eds) Biological Approaches to Rational Drug Design. CRC Press, Ann Arbor, 1994.

74. Zopf, D. and Roth, S. Oligosaccharides as anti-infective drugs [Review]. Lancet, 347: 1017-1027 , 1996.

75. Zopf, D., Simon, P.M., Barthelson, R., Cundell, D., Idänpään-Heikkilä, I., Tuomanen, E.: Development of antiadhesion carbohydrate drugs for clinical use. In Kahane, I. and Ofek, I. (eds) Toward Anti-adhesion Therapy for Microbial Diseases, Plenum, NY pp. 35-38, 1996.

76. Simon, P.M., Goode, P.L., Mobasseri, A., and Zopf, D. Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. Infect. Immun. 65: 750-757, 1997.

77. Idänpään-Heikkilä, I., Simon, P.M., Zopf, D., Vullo, T., Cahill, P., Sokol, K., and Tuomanen, E. Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. J. Infect. Diseases 176: 704-12, 1997.

78. Leickt, L., Bergström, M., Zopf, D., and Ohlson, S. Bioaffinity chromatography in the 10 mM range of Kd. Anal. Biochem. 253: 135-6, 1997.

79. Barthelson, R., Mobasseri, A., Zopf, D., and Simon, P. Adherence of *Streptococcus pneumoniae* to respiratory epithelial Cells is inhibited by sialylated oligosaccharides. Infect. Immun. 66: 1439-1444, 1998.

80. Simon, P.M., Neethling, F.A., Taniguchi, S., Goode, P.L., Zopf, D., Hancock, W.W., and Cooper, D.K.C. Intravenous infusion of Galα1-3Gal oligosaccharides in baboons delays hyperacute rejection of porcine xenografts. Transplantation 65: 346-53, 1998.

81. Ohlson, S., Bergstrom, M., Leickt, L., and Zopf, D. Weak affinity chromatography of small sacccharides with immobilised wheat germ agglutinin and its application to monitoring of carbohydrate transferase activity. Bioseparation 7: 101-105, 1998

82. Fu, D., and Zopf, D. Analysis of sialyllactoses in blood and urine by high-performance liquid chromatography. Anal. Biochem. 269: 113-23, 1999.

83. Nakhla, T., Fu, D., Zopf, D., Brodshy, N.L., and Hurt, H. Neutral oligosaccharide content of preterm human milk. Brit. J. Nutr. 82: 361-7, 1999.

84. Mysore, G.V., Wigginton T., Simon, P.M., Zopf, D, Heman-Ackah L.M., and Dubois A. Treatment of *Helicobacter pylori* infection in rhesus monkeys using a novel antiadhesion compound. Gastroenterology 117: 1316-25, 1999

85. Simala-Grant, J. L., Zopf, D., and Taylor, D.E. Antibiotic susceptibility of attached and free-floating Helicobacter pylori. J. Antimicrob Chemo 47: 555-563, 2001

ETH Eidgenössische Technische Hochschule Zürich
Ecole polytechnique fédérale de Zurich
Politecnico federale di Zurigo
Swiss Federal Institute of Technology Zurich

Professor Dr. James E. Bailey
Institute of Biotechnology

ETH Zürich
CH 8093 Zürich,
Switzerland

Tel.: 41-1-633 31 70
41-1-633 34 03
FAX: 41-1-633 10 51

Paul Simon, Ph.D.
V. P. for Research
VIMRx Pharmaceuticals, Inc.
2751 Centerville Rd., Suite 210
Wilmington, DE 19808
USA

FAX: +001 302 998-3794

Zurich, 22 February 1999

Dear Dr. Simon,

Thank you for your email message concerning antibody glycosylation. We do not anticipate any particular problems in implementing our glycosylation engineering strategy for improving antibody ADCC activity in large scale, high production cultures.

Of course, you are correct that glycosylation can be affected by process changes. In fact, we have prepared a comprehensive review of glycosylation, focusing more on genetic changes but also mentioning the process-related ones that have been reviewed recently elsewhere. I will send you a copy of this review by mail, since it is somewhat long. Unfortunately, it is available only in a book published as a conference preceding, so is not so widely accessible.

In general, I think that the process complications and costs associated with producing and utilizing a glycosyltransferase and also supplying the donor substrate make the exogenous manipulation of glycosylation generally far less attractive than engineering the cells to maximize the production of the desired glycoform. Also, you will see in the N-glycosylation pathway given in our paper that only one particular glycoform would be a suitable substrate for arriving at the desired final product, greatly reducing the potential yield of such exogenous glycosylation manipulation. Failure of much simpler cofactor-requiring enzyme catalyzed reactions to gain industrial success in competition with whole-cell biocatalysis speaks very strongly in my opinion against the competitive prospects of *in vitro* remodeling of glycosylation. I think it is much better to do this in the cells.

Best wishes,

J Bailey

Neose Signs R&D and Licensing Agreement with Wyeth-Ayerst

First Commercial Use of GlycoAdvance – Novel Protein Glycosylation Technology

Horsham, PA, December 19, 2001 – Neose Technologies, Inc. (NasdaqNM: NTEC) today announced that it has entered into a research, development and license agreement with Wyeth-Ayerst Laboratories, the pharmaceutical division of American Home Products Corporation (NYSE: AHP), for the use of Neose's GlycoAdvance™ technology to develop an improved production system for Wyeth's biopharmaceutical compound, rPSGL-Ig (P-selectin glycoprotein ligand). rPSGL-Ig is a P-selectin antagonist that is being developed to treat inflammation and thrombosis associated with acute coronary syndrome and reperfusion injury. It is currently being evaluated in Phase II clinical trials in patients being treated for heart attack. Wyeth is evaluating the use of GlycoAdvance in the production of rPSGL-Ig for Phase III clinical trials and commercial launch.

Neose will develop processes for the commercial-scale manufacture of proprietary enzymes and sugar nucleotides to be used in the production of rPSGL-Ig, and will license GlycoAdvance to Wyeth for commercial production of the drug. During commercial production of Wyeth's current rPSGL-Ig, Neose will receive ongoing payments tied to yield improvements achieved using GlycoAdvance in the production of rPSGL-Ig. In addition, Wyeth has the option to use GlycoAdvance to develop a next generation rPSGL-Ig, in which case Neose would receive royalties on product sales.

Under the agreement, Neose will receive license, research, and milestone payments that would total up to $17 million if all milestones are met. In addition to ongoing product payments, Neose and Wyeth will also enter into a supply agreement for the long-term supply of GlycoAdvance process reagents.

"We welcome Wyeth as our first commercial partner for GlycoAdvance and look forward to contributing to the success of rPSGL-Ig," says Stephen Roth, Ph.D., Neose's Chairman and CEO. "Wyeth and Neose have worked together extensively to show that GlycoAdvance can be applied to a drug in late stage clinical development. We are particularly excited to be working with Wyeth, given their significant investment in biopharmaceutical drug development, and their commitment to being a world leader in biologics manufacturing."

"GlycoAdvance gives us an important competitive advantage that complements our substantial and growing capital investment in manufacturing capacity," says L. Patrick Gage, Ph.D., President, Wyeth-Ayerst Research. "Using GlycoAdvance with rPSGL-Ig will help us launch the drug with manufacturing capacity in place to supply the projected needs of our initial indication, while giving us the flexibility to supply additional indications as they are developed."

Background on GlycoAdvance

There are more than 360 biotechnology drugs in development for more than 200 diseases. Many of these drugs are glycoproteins - proteins and antibodies that include complex carbohydrates, or sugar chains, as an integral part of their structure. In 2000, worldwide sales of protein and antibody drugs were about $20 billion. By 2010 worldwide sales are expected to exceed $90 billion. Many of these drugs will be glycoproteins and may be appropriate candidates for GlycoAdvance.

GlycoAdvance is Neose's proprietary enzymatic technology for completing the carbohydrate chains on glycoproteins after they have been produced in a biological expression system such as Chinese hamster ovary (CHO) cells. GlycoAdvance uses a class of enzymes, glycosyltransferases, to add individual sugar units onto the carbohydrate structures on glycoproteins. GlycoAdvance can be used with glycoprotein therapeutics, including fusion proteins and monoclonal antibodies, to extend half-life, increase effectiveness and improve manufacturing efficiency.

Glycoproteins contain complex carbohydrate structures attached to the protein portion of the molecule. These carbohydrates are integral to the structure and function of a glycoprotein and help determine how long the drug stays active in the body. Incomplete carbohydrate structures can result in the drug being cleared from the body too quickly, or may result in the drug being less effective. This means that a greater amount of the drug may be required to achieve the intended effect.

Achieving and maintaining the proper carbohydrate structures on glycoproteins is a major challenge in biotechnology manufacturing. Recombinant therapeutic glycoproteins are produced in living cells, usually CHO cells. The use of cell systems to produce glycoproteins requires balancing the cells' ability to produce protein with their ability to put on the required carbohydrates. As the cells' protein output increases, they do not maintain

the proper level of carbohydrates. This often results in low yields of usable product that adds to the cost and complexity of producing these drugs. These low yields are a significant contributor to the critical worldwide shortage of biologics manufacturing capacity.

Background on rPSGL-Ig

Wyeth's rPSGL-Ig is a recombinant version of the human PSGL-1 glycoprotein, linked to the Fc portion of a human antibody. PSGL-1 glycoprotein extends from the surface of white blood cells, or leukocytes, and helps the cells bind to the blood vessel wall in a process known as cell adhesion. PSGL-1 plays a critical role in the migration of these cells from the bloodstream to the site of tissue damage. This is an essential process in helping the body heal itself after an injury. However, in some instances, it can be harmful. Immediately following a heart attack, the leukocytes that attach to the damaged blood vessels exacerbate local inflammation that causes additional tissue damage.

rPSGL-Ig protects the site of tissue damage by preventing leukocytes and platelets from adhering and causing inappropriate inflammation and/or thrombosis. rPSGL-Ig is in Phase II clinical trials evaluating its ability to help accelerate clot dissolution and prevent reperfusion injury following a heart attack. rPSGL-Ig may also have use in solid organ transplantation and arterial vascular diseases including stroke.

About Neose Technologies

Neose develops proprietary technologies for the synthesis and manufacture of complex carbohydrates. The company uses its broad technology platform in the following programs: GlycoAdvance for correcting incomplete or incorrect glycosylation encountered in the manufacture of recombinant glycoproteins; GlycoTherapeutics to develop and produce novel carbohydrate-based therapeutics; and GlycoActives to develop and produce novel carbohydrate-based food ingredients.

Conference Call/Webcast

A conference call and webcast will be held for the investment community on Thursday, December 20, 2001, at 8:30 a.m. EST. The dial-in number for domestic callers is 800-967-7140. The dial-in number for international callers is 719-457-2629. A replay of the call will be available for 7 days beginning approximately four hours after the call's conclusion. The replay number for domestic callers is 888-203-1112 using the passcode 421599. The replay number for international callers is 719-457-0820, also using the passcode 421599. Live audio of the conference call will be simultaneously broadcast over the Internet through World Investor Link's Vcall website, located at www.vcall.com. To listen to the live call, please go to the web site at least fifteen minutes early to register, download, and install any necessary audio software. For those who cannot listen to the live broadcast, a replay will be available shortly after the call.

CONTACTS:

Neose Technologies, Inc.

Sr. Director, Corporate Communications

(215) 773-1802 Direct

(215) 441-5890 Main

(215) 285-1244 Cell

(610) 902-7336

Jason Rubin

(610) 941-2741

For more information, please visit www.neose.com.

"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release that are not historical facts are "forward-looking statements" that involve risks and uncertainties. Among the risks are that the development of rPSGL-Ig using GlycoAdvance may not succeed, or rPSGL-Ig may not receive regulatory approval or be commercialized successfully. For a more detailed discussion of these risks and uncertainties, any of which could cause the Company's actual results to differ from those contained in any forward-looking statement, see the "Risk Factors" section of Item 1 of the Company's Annual Report on Form 10-K for the year ended December 31, 2000.

Neose Technologies, Inc.

Neose Informed of Wyeth's Intention to Discontinue Development of rPSGL-Ig for Myocardial Infarction

Decision Unrelated to Neose's GlycoAdvance™

HORSHAM, PA, May 9, 2002 – Neose Technologies (NasdaqNM: NTEC) announced that it has been informed today by Wyeth Pharmaceuticals (NYSE: WYE) that Wyeth does not intend to continue clinical development of their compound, rPSGL-Ig, for myocardial infarction due to disappointing results in Phase II clinical trials. This decision was unrelated to the performance of Neose's GlycoAdvance technology. Although it is possible that development of rPSGL-Ig may continue for other indications, the timing or likelihood of continued development is not known.

Boyd Clarke, president and CEO of Neose Technologies, said, "We knew this compound was in Phase II clinical trials, and was subject to the normal risks and uncertainty associated with clinical drug development. Although our technology was working as planned, we are disappointed by the news from Wyeth. We hope to continue working with Wyeth to use GlycoAdvance in their therapeutic protein development programs."

Neose develops proprietary technologies for the synthesis and manufacture of complex carbohydrates. The company uses its broad technology platform in the following programs: GlycoAdvance products and services for correcting incomplete or incorrect glycosylation encountered in the manufacture of recombinant glycoproteins; GlycoTherapeutics™ to develop and produce novel carbohydrate-based therapeutics; and GlycoActives™ to develop novel carbohydrate-based food and nutritional ingredients.

CONTACTS:

Neose Technologies, Inc.

Matt Kremer

Sr. Director, Corporate Communications

A. Brian Davis

Acting Chief Financial Officer

(215) 315-9000

E-mail: info@neose.com

For more information, please visit www.neose.com.

"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release that are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a more detailed discussion of these risks and uncertainties, any of which could cause the Company's actual results to differ from those contained in any forward-looking statement, see the "Risk Factors" section of Item 1 of the Company's Annual Report on Form 10-K for the year ended December 31, 2001.

Neose Technologies and MacroGenics Sign Research Collaboration and License Agreement

HORSHAM, Pa., and ROCKVILLE, Md., April 26 /PRNewswire-FirstCall/ -- Neose Technologies, Inc. (Nasdaq: NTEC) and MacroGenics Inc. announced today that the companies have entered into a research collaboration and license agreement on multiple monoclonal antibodies. Neose will apply its GlycoAdvance(TM) and GlycoPEGylation(TM) technologies to MacroGenics compounds with the goal of improving the therapeutic properties of these proteins.

MacroGenics has the right to take a limited number of remodeled compounds into development. Following the initial research phase, MacroGenics will be responsible for funding the further development of these licensed compounds under an exclusive license from Neose. In exchange, Neose will be entitled to receive various option fee, milestone, and royalty payments as products are developed and commercialized under the agreement.

"We are impressed with MacroGenics' expertise in the antibody field and look forward to working with them. They have important new technology for the development and modification of monoclonal antibodies, particularly in the Fc region, and we believe that combining our technologies may yield more effective new treatments for chronic diseases," said C. Boyd Clarke, Neose president, chief executive officer and chairman.

"We are excited by the potential therapeutic improvements that can be made to our monoclonal antibodies utilizing Neose's GlycoAdvance and GlycoPEGylation technologies," said Scott Koenig, M.D., Ph.D., president and chief executive officer of MacroGenics.

Neose is a biopharmaceutical company focused on the improvement of protein therapeutics through the application of its proprietary technologies. By applying its GlycoAdvance and GlycoPEGylation technologies, Neose is developing proprietary protein drugs that are improved versions of currently marketed therapeutics with proven efficacy. These second generation proteins are expected to offer significant advantages, such as less frequent dosing and improved safety and efficacy. In addition to developing its own products or co-developing products with others, Neose is entering into strategic partnerships for the inclusion of its technologies into products being developed by other biotechnology and pharmaceutical companies.

MacroGenics is a privately funded company focused on the development, manufacture and commercialization of biotechnology products including immunotherapeutics for cancer, infectious diseases, and autoimmune disorders. MacroGenics' core platform involves antibody receptor-related technologies which are employed to improve the ways cytotoxic antibodies mediate cell killing for the treatment of cancers and to prevent autoantibodies from triggering disease in autoimmunity.

CONTACTS:

Neose Technologies, Inc.
Robert I. Kriebel
Sr. Vice President and Chief Financial Officer

Barbara Krauter
Manager, Investor Relations
(215) 315-9000

For more information, please visit www.neose.com.

MacroGenics Inc.
Michael Richman
Exec. Vice President and Chief Operating Officer
(301) 251-5172

For more information, please visit www.macrogenics.com.

"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release regarding our business that are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a discussion of these risks and uncertainties, any of which could cause our actual results to differ from those contained in the forward-looking statement, see the section of Neose's Annual Report on Form 10-K for the year ended December 31, 2003, entitled "Factors Affecting the Company's Prospects" and

discussions of potential risks and uncertainties in Neose's subsequent filings with the SEC.

SOURCE Neose Technologies, Inc.

Neose Technologies, Inc. and Monsanto Protein Technologies Sign Research Agreement

HORSHAM, Pa., and ST. LOUIS, Nov. 25 /PRNewswire-FirstCall/ -- Neose Technologies, Inc. (Nasdaq: NTEC) and Monsanto Protein Technologies, a unit of Monsanto Company (NYSE: MON), today announced that they have entered into a research agreement to investigate the use of Neose's GlycoAdvance(TM) technology to enhance the glycosylation of therapeutic monoclonal antibodies produced in plants.

Today, the majority of therapeutic monoclonal antibodies are produced by mammalian cell culture. The glycosylation patterns of plant-produced monoclonal antibodies differ significantly from monoclonal antibodies produced by mammalian cell culture. Monoclonal antibodies produced in plants have incomplete glycosylation patterns, resulting in the inability to activate the complement system and other types of critical effector function. This research will combine Monsanto Protein Technologies' expertise in transgenic plant production of monoclonal antibodies with Neose's expertise in glycosylation. This is expected to enhance the ability of plant-produced monoclonal antibodies to initiate complement activation.

"Monsanto believes this science promises tremendous benefits and will someday provide greater access to life-saving therapeutic drugs, thus providing more options for patients and doctors," said Cheryl Morley, President of Monsanto's Animal Ag and Protein Technologies Group. "We are excited about working with Neose and the GlycoAdvance technology."

"GlycoAdvance has the potential to make an important contribution to the glycosylation, and therefore the therapeutic usefulness of plant-produced proteins. We are delighted to be working with Monsanto, a leader in the development of plant-based systems," said C. Boyd Clarke, Neose president and chief executive officer.

Monsanto Protein Technologies, a unit of Monsanto, is focused on contract manufacturing of therapeutic proteins at very large scale derived from plant biotechnology. Monsanto is recognized as a leader in plant biotechnology and recombinant protein technology. By leveraging this breadth of experiences ranging from genomics and seed breeding, to large scale sterile protein manufacturing, Monsanto Protein Technologies can deliver a cost-effective process for producing therapeutic proteins at very large scale.

Neose develops proprietary technologies for using enzymes to manufacture complex carbohydrates. Neose is using its broad technology base to develop novel and improved products for itself and its partners, primarily focusing on protein therapeutics. Neose markets its technology for improving protein drugs under the name GlycoAdvance. GlycoAdvance is used to modify the human carbohydrate structures on therapeutic glycoproteins. Neose is also developing its technology to create novel glycosylation patterns, and to link other molecules, such as polyethylene glycol, to glycoproteins. The application of this technology to proteins potentially results in improved clinical activity and pharmacokinetic profile, enhanced drug development flexibility, stronger and additional patent claims, and yield improvements.

For more information pertaining to Neose Technologies, Inc., please visit http://www.neose.com.

For more information pertaining to Monsanto Protein Technologies, please visit http://www.mpt.monsanto.com.

Cautionary Statements Regarding Forward-Looking Information of

Neose Technologies, Inc.:

Statements in this press release regarding our business that are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a discussion of these risks and uncertainties, any of which could cause our actual results to differ from those contained in the forward-looking statement, see the "Risk Factor" section of Item 1 of our Annual Report on Form 10-K for the year ended December 31, 2001.
Cautionary Statements Regarding Forward-Looking Information of Monsanto Company and Monsanto Protein Technologies (a unit of Monsanto Company):

Certain statements contained in this release, such as statements concerning the anticipated financial results, current and future product performance, regulatory approvals, currency impact, business and financial plans and other non-historical facts pertaining to Monsanto Company or Monsanto Protein Technologies, Inc. are "forward-looking statements." These statements are based on current expectations and currently available information. However, since these statements are based on factors that involve risks and uncertainties, the actual performance and results of Monsanto Company or Monsanto Protein Technologies, Inc. may differ materially from those described or implied by such forward-looking statements. Factors that could cause or contribute to such

differences include, among others: the success of the research and development activities of Monsanto Company and Monsanto Protein Technologies, Inc. and the speed with which regulatory authorizations and product launches may be achieved; the ability of Monsanto Company or Monsanto Protein Technologies, Inc. to successfully market new and existing products in new and existing domestic and international markets; the ability of Monsanto Company or Monsanto Protein Technologies, Inc. to achieve and maintain protection for its respective intellectual property; the exposure of Monsanto Company or Monsanto Protein Technologies, Inc. to lawsuits and other liabilities and contingencies, including those related to intellectual property, product liability, regulatory compliance, environmental contamination and antitrust; and other risks and factors detailed in the Monsanto Company's filings with the U.S. Securities and Exchange Commission. Undue reliance should not be placed on these forward-looking statements, which are current only as of the date of this release. Monsanto Company and Monsanto Protein Technologies, Inc. disclaim any current intention to revise or update any forward-looking statements or any of the factors that may affect actual results, whether as a result of new information, future events or otherwise.

CONTACTS:

Neose Technologies, Inc.
Robert I. Kriebel
Sr. Vice President and
Chief Financial Officer
215-315-9000

Barbara Krauter
Investor Relations Associate
215-315-9000

Monsanto Protein Technologies
Lee Quarles
Monsanto Public Affairs
314-694-4452

Jon McIntyre, Ph.D.
Chief Scientist
314-694-8428

SOURCE Neose Technologies, Inc.

Neose and Novo Nordisk Sign Research and Development Collaboration Agreement

HORSHAM, Pa., Oct. 1 /PRNewswire-FirstCall/ --
Neose Technologies, Inc. (Nasdaq: NTEC) today announced that it has entered into a research and development collaboration agreement with Novo Nordisk A/S (NYSE: NVO) for the use of Neose's GlycoAdvance™ technology to make clinically significant improvements to a Novo Nordisk therapeutic protein.

We believe Neose's GlycoAdvance technology may make important improvements to marketed therapeutic proteins in the Novo Nordisk pipeline. As a global leader in the development and commercialization of biological products, Novo Nordisk is an ideal partner for GlycoAdvance. We are excited by the potential opportunities to add value to their products through this collaboration, said C. Boyd Clarke, Neose president and chief executive officer.

Novo Nordisk is a focused healthcare company and the world leader in diabetes care. In addition, Novo Nordisk has a leading position within areas such as haemostasis management, growth hormone therapy and hormone replacement therapy. Novo Nordisk manufactures and markets pharmaceutical products and services that make a significant difference to patients, the medical profession and society. With headquarters in Denmark, Novo Nordisk employs approximately 17,900 people in 68 countries and markets its products in 179 countries. For further company information visit www.novonordisk.com.

Neose develops proprietary technologies for using enzymes to manufacture complex carbohydrates. Neose is using its broad technology base to develop novel and improved products for itself and its partners, primarily focusing on protein therapeutics. Neose markets its technology for improving protein drugs under the name GlycoAdvance™. We use GlycoAdvance™ to modify the human carbohydrate structures on therapeutic glycoproteins. We are also developing our technology to create novel glycosylation patterns, and to link other molecules, such as polyethylene glycol, to glycoproteins. The application of this technology to proteins potentially results in improved clinical activity and pharmacokinetic profile, enhanced drug development flexibility, stronger and additional patent claims, and yield improvements. We are exploring the use of our technology to enable the development of carbohydrate-based therapeutics, and the development of novel carbohydrate food and nutritional ingredients.

CONTACTS:

Neose Technologies, Inc.

Robert I. Kriebel

Sr. Vice President and Chief Financial Officer

Barbara Krauter

Investor Relations Associate

(215) 315-9000

For more information, please visit www.neose.com.

Safe Harbor Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release regarding our business that are not historical facts are forward-looking statements that involve risks and uncertainties. For a discussion of these risks and uncertainties, any of which could cause our actual results to differ from those contained in the forward-looking statement, see the Risk Factor section of Item 1 of our Annual Report on Form 10-K for the year ended December 31, 2001.

SOURCE Neose Technologies, Inc.

/CONTACT: Robert I. Kriebel, Sr. Vice President and Chief Financial Officer, or Barbara Krauter, Investor Relations Associate, both of Neose Technologies, +1-215-315-9000/

/Web site: http://www.novonordisk.com /

/Web site: http://www.neose.com /

(NTEC NVO)

CO: Neose Technologies, Inc.; Novo Nordisk A/S
ST: Pennsylvania, Denmark
IN: MTC BIO HEA
SU: JVN CON

FB
-- PHTU058 --
4515 10/01/2002 16:01 EDT http://www.prnewswire.com